Human CIC-3 Is Not the Swelling-activated Chloride Channel Involved in Cell Volume Regulation*

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Volume regulation is essential for normal cell function. A key component of the cells’ response to volume changes is the activation of a channel, which elicits characteristic chloride currents (ICl, Swell). The molecular identity of this channel has been controversial. Most recently, CIC-3, a protein highly homologous to the CIC-4 and CIC-5 channel proteins, has been proposed as being responsible for ICl, Swell (1). Subsequently, however, other reports have suggested that CIC-3 may generate chloride currents with characteristics clearly distinct from ICl, Swell. Significantly different tissue distributions for CIC-3 have also been reported, and it has been suggested that two isoforms of CIC-3 may be expressed with differing functions. In this study we generated a series of cell lines expressing variants of CIC-3 to rigorously address the question of whether or not CIC-3 is responsible for ICl, Swell. The data demonstrate that CIC-3 is not responsible for ICl, Swell and has no role in regulatory volume decrease, furthermore, CIC-3 is not activated by intracellular calcium and fails to elicit chloride currents under any conditions tested. Expression of CIC-3 was shown to be relatively tissue-specific, with high levels in the central nervous system and kidney, and in contrast to previous reports, is essentially absent from heart. This distribution is also inconsistent with the previous proposed role in cell volume regulation.

Although a cell swelling-activated chloride current (ICl, Swell) required for RVD has been carefully characterized in several cell types, the molecular identity of the channel has not yet been established (3–5). This is primarily due to three experimental limitations. First, the current is ubiquitous, such that cell lines exhibiting little or no current necessary for expression cloning are not available. Second, no specific, high affinity blockers of the current are known. Third, the magnitude and the rate of activation of the currents are readily perturbed by both endogenous and exogenous factors making quantitative analysis difficult. The latter issue is illustrated by the fact that P-glycoprotein, proposed as a candidate for the cell swelling-activated chloride channel, was subsequently shown to be a regulator of endogenous channel activity (6, 7), whereas another candidate, pICln, was also shown not to be the channel and its precise role is still uncertain (8, 9). Another swelling-activated chloride channel expressed in many cell types, CIC-2, has also been suggested as contributing to RVD (10). However CIC-2 generates a current with biophysical and pharmacological characteristics that differ significantly from ICl, Swell (11–13), and in a human intestinal epithelial cell line it has been shown that CIC-2 does not contribute to RVD (13).

Recently, a series of studies has led to the suggestion that CIC-3 is the swelling-activated chloride channel responsible for ICl, Swell. The gene coding for CIC-3 was first cloned from rat by a homology-based cloning strategy; its predicted amino acid sequence is similar to other CIC channels (14, 15). The human cDNA was subsequently cloned and sequenced from fetal brain (16), and the guinea pig version was cloned and sequenced from cardiac myocytes (1). Expression of gpCIC-3 was reported to increase significantly ICl, Swell (1, 17). This view was supported by antisense experiments where reduction of CIC-3 expression was reported to decrease ICl, Swell (18).

Subsequently, the role of CIC-3 in ICl, Swell has been challenged. Attempts to replicate experiments with gpCIC-3 and hCIC-3, either in Xenopus oocytes or in mammalian cell lines (HEK293 and NIH3T3), were unsuccessful (19). In another study, expression of rCIC-3 in CHO cells was reported to generate a Ca2+-sensitive chloride channel (15). Further complexity was introduced by the suggestion that short and long versions of CIC-3 could potentially be generated in vivo using two different translation initiation sites, identical except for an additional 58 amino acids at the N-terminal of the long version (20). It was reported that expression of the short and long versions generated, in Chinese hamster ovary-K1 cells, distinct currents that are CIC-5-like and ICl, Swell-like, respectively (20), although the ion selectivity of the CIC-5-like currents is reported both as $\Gamma^- > Cl^-$ and $Cl^- > \Gamma^-$ (21).

The maintenance of a constant cell volume in the face of fluctuating intra- and extracellular osmolarity is essential for normal cell function. Following cell swelling upon exposure to hypotonic solution, animal cells restore their volume toward its original value by activation of channels and transporters in the plasma membrane: the loss of K+ and Cl– ions and organic osmolytes, followed by obligatory loss of water, leads to regulatory volume decrease (RVD)† (2).
The tissue distribution of CIC-3 is also ambiguous. Kawasaki et al. (14) reported mRNA coding for CIC-3 mainly in brain and kidney, but not in heart. In contrast, Shimada et al. (20) reported high levels of expression of CIC-3 protein in the liver, and Britton et al. (22) high levels in heart. Furthermore, CIC-3 was reported in bovine non-pigmented ciliary cells to be localized mainly to the nucleus (18), yet to the canalicular membrane in hepatocytes (20).

To resolve the controversy surrounding CIC-3 we generated cell lines expressing the long and short versions of hCIC-3-GFP, with hCIC-5-GFP as control, in HEK293 cells. Neither the long nor the short version of hCIC-3-GFP affected the swelling-activated currents significantly or influenced cell volume regulation. Indeed, CIC-3 generated no detectable chloride currents when overexpressed in response to change in cellular Ca2+ concentrations.

The experiments presented here exclude CIC-3 as the channel responsible for I\textsubscript{Cl,Swell} and cell volume regulation.

**EXPERIMENTAL PROCEDURES**

**Generation of Cell Lines Expressing hCIC-3-GFP and hCIC-5-GFP.** The original cDNA sequence coding for ClC-3 predicted a protein of 760 amino acids (the short version) (14). Subsequently, an upstream ATG codon was identified, and a protein with an additional 58 amino acids at the N terminus was predicted (the long version) (20). It has been suggested that both versions may be expressed (20). To study the localization of hCIC-3, we generated cell lines expressing both the short and long versions fused to GFP at the C terminus. It has previously been reported that GFP tags at the C terminus of CIC channels do not impair function (23). As a control for function, a cell line expressing hCIC-3 with no GFP tag was also generated.

Plasmids containing overlapping cDNA fragments encoding hCIC-3 were obtained from Dr. Borsani (16). Appropriate restriction fragments were excised from these plasmids, or generated from these plasmids by PCR, and ligated together to generate a full-length CLCN3 cDNA. The PCR primer at the 5′-end (5′-ATTGTGGACCTAGCCACATGAAATGGAGGCACGACG-3′) was designed to introduce a consensus Kozak sequence and a unique NheI restriction site upstream of the start codon. The full-length cDNA was inserted as a NheI-Xhol fragment into the expression vector pCIneo (Promega) to generate the expression plasmid pCI-CIC-3. The cDNA was verified by sequencing. To generate the long version, the PCR amplification was cloned from human primary lung fibroblasts by a reverse transcriptase reaction (Omniscript, Qiagen) using random hexamer primers (Roche Molecular Biochemicals), followed by PCR using primers specific for CIC-3: the upstream promoter was 5′-CGGATATGCTGATCCACCATGACCACGAGTGTCCAT-3′ and at the downstream promoter 5′-AGAACGCTTGAATGATCCAGTGACTTCGTT-3′.

The PCR fragment was ligated as an NheI-BamHI fragment into pCIneo-Xhol. This had been modified by PCR using primer 5′-AGAACTGTTAATGGATCCTCCATTTGT-3′ (BamHI site) together with 5′-AGTTGGAACGCTAGCCACCATGACAAATGGAGGCAGC-3′ (NheI site) and verified by sequencing. To generate the long version, the missing NheI restriction site upstream of the start codon. The resultant plasmid was designated pCI-ClC-3long.

**Biotinylolation of Membrane Proteins**—Cells grown on poly-L-lysine-coated 75-cm\textsuperscript{2} flasks or 35-mm dishes to 80% confluency were incubated in 5 ml of reducing buffer (50 mM dithiothreitol, 2% SDS, 0.1 M glycerol, 5% 3-mercaptoethanol, 2% SDS, 0.05% bromophenol blue) for 10 min at 90 °C and analyzed by Western blotting.

**Protein Preparation and Western Blotting**—Cell extracts for Western blotting were prepared by detaching cells with PBS containing 5 mM EDTA followed by lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40) with protease inhibitors (Roche Molecular Biochemicals). The lysate was sonicated to shear the DNA.

**Confocal Immunofluorescence Imaging**—Cells, grown on glass coverslips with poly-L-lysine, were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) with calcium and magnesium. For immunocytochemistry, the cells were permeabilized in 0.1% Triton X-100 for 10 min on ice.

**Generation of Cell Lines Expressing hClC-3—**Generation of cell lines expressing hClC-3-GFP and hClC-5-GFP—

**For electrophoresis, samples were mixed with equal amounts of reducing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40) with protease inhibitors (Roche Molecular Biochemicals).** The lysate was sonicated to shear the DNA.
Electrophysiology—Chloride currents were measured in whole cell recording mode of the patch-clamp technique as described previously (7). To prevent voltage offset when bath Cl− concentration was changed, the bath reference electrode was connected through an agar bridge that maintains a constant Cl− concentration in the immediate vicinity of the Ag/AgCl electrode. The extracellular (bathing) isotonic solution contained 100 mM NMDGCl, 0.5 mM MgCl2, 1.3 mM CaCl2, 10 mM HEPES titrated to pH 7.4 with Tris. The osmolality was corrected to 300 mOsm with mannitol. The extracellular hypotonic solution, used to elicit swelling-activated currents, had the same composition as the isotonic solution except that the osmolality was corrected with mannitol to 220 mOsm. Extracellular solutions with chloride substitution were obtained by replacing 100 mM NMDGCl with 100 mM of the respective anion salts (NaCl, NaF, or NaBr), and osmolality was adjusted with mannitol to 220 or 300 mOsm for hypotonic or isotonic solutions, respectively. The intracellular (pipette) solution was 100 mM NMDGCl, 1.2 mM MgCl2, 1 mM EDTA, 10 mM HEPES, 2 mM ATP titrated to pH 7.4 with Tris, and osmolality was adjusted to 280 mOsm with mannitol. After extensive washing with isotonic solution not containing calcium, the cells were then transferred to polyvinylidene difluoride membranes coated coverslips and loaded, before the experiment, for 5 min with 2.5 μM Calcein-AM (Molecular Probes) in isotonic solution (70 mM NaCl, 5 mM KCl, 0.5 mM MgCl2, 2 mM CaCl2, 5.5 mM glucose, 10 mM Hepes buffer, pH 7.4, and osmolality was adjusted to 320 mOsm with mannitol). After extensive washing with isotonic solution not containing calcein-AM, cells were transferred to a perfusion chamber set on the stage of the microscope. Experiments were performed at room temperature. Cell volume measurements were performed by non-paired t test; statistical significance was accepted for p < 0.05(*) or p < 0.01(**). The data above do not exclude the possibility that the protein is located in limited horizontal resolution of confocal microscopy, the data shown were representative of the large majority of the cells within a single clone and of several independent clones expressing each GFP fusion protein (data not shown).

CIC-3 Is Present on the Plasma Membrane—The data above suggest that a proportion of the CIC-3-GFP fusion protein is localized to the plasma membrane. However, because of the limited horizontal resolution of confocal microscopy, the data do not exclude the possibility that the protein is located in vesicles just beneath the plasma membrane. To demonstrate that the CIC-3-GFP fusion proteins are inserted in the plasma membrane, cells were labeled with the membrane-impermeant, thiol-reactive reagent biotin-maleimide (28). The biotinylated proteins were then isolated using immobilized neovavidin, and any GFP fusion proteins in the biotinylated protein fraction were detected by SDS-PAGE and Western blotting using anti-GFP antibodies. In the absence of biotinylation, no proteins were bound by the resin, showing that the labeling is specific (data not shown). A significant proportion of each of the GFP fusion proteins tested was accessible to biotin maleimide in intact cells, consistent with a plasma membrane location (Fig. 3, top panel a). Under these conditions, an intracellular control antigen known to be located just beneath the plasma membrane, the Ras pathway protein SHC (29) was not labeled, demonstrating that the biotin maleimide does not permeate the plasma membrane (see “Experimental Procedures”).
membrane (Fig. 3, bottom panel a). When cell membranes were permeabilized with saponin (Fig. 3b), there was, as expected, a significant increase in labeling of CIC-3-GFP proteins (top panel b), and SHC was now also labeled (bottom panel b). Similar results were obtained also with the clone expressing the native CIC-3 protein (data not shown). Thus, in the HEK cell lines a significant proportion of both the hCIC-3 and hCIC-5-GFP fusion proteins is located in the plasma membrane.

hCIC-3 Is Not a Swelling-activated Channel—Having established that the hCIC-3GFP fusion proteins were present in the plasma membrane, cell swelling-activated chloride currents were studied under the whole cell configuration of the patch clamp technique. Cells were maintained at the holding potential of 0 mV and stimulated by a standard protocol from −80 mV to +120 mV in 40-mV steps. Fig. 4 shows representative current traces in isotonic solution (upper panels), and 7 min after exposure to a 30% hypotonic solution (lower panels) at which time the currents had reached steady state (Fig. 5a). Very low currents were recorded in isotonic conditions (Fig. 5b) except for cells expressing hCIC-5. Following hypo-osmotic shock, all cell lines, including the parental HEK293 cells, exhibited a similar current, showing moderate outward rectification, fast activation, and time and voltage inactivation at +120 mV (Fig. 4, bottom panel). No statistically significant difference in either the magnitude (Fig. 5c) or rate of activation of I_{Cl, Swell} was observed between the different lines. Osmotically induced currents were blocked by addition of 10 μM tamoxifen, an inhibitor of swelling-activated chloride channel (30) (data not shown).

Although data with other CIC channels show that a GFP tag at the C terminus has no effect on channel properties (23), we also confirmed that no difference in current magnitude or current characteristics were observed between HEK293 cells expressing CIC-3 and CIC-3-GFP fusions (Figs. 4 and 5).

Previous studies (1) have suggested that the N579K mutation of CIC-3 suppresses outward rectification of the current and changes its halide selectivity. Expression of hCIC-3(N579K)-GFP induced no change in either the magnitude of swelling-activated chloride currents (Fig. 5c) or its outward rectification (Fig. 4). The anion selectivity of currents recorded in cells expressing the mutated protein (Fig. 6) did not change when compared with the currents recorded from cells expressing the wild type protein: for all cell lines tested the halide permeability was: I− ∼ Br− > Cl− > F− (Fig. 6).

In conclusion, neither the magnitude, rate of activation, nor characteristics of swelling-activated currents in HEK293 cells were affected by overexpression of any of the versions of hCIC-3 tested. This is despite the fact that we demonstrated that the proteins were localized to the plasma membrane and that similar expression of a closely related protein, hCIC-5 (see below), generates characteristic currents. These data provide strong evidence that CIC-3 as expressed has no channel activity.

Expression of CIC-3 Does Not Affect RVD—To further test the involvement of CIC-3 in volume regulation, we measured RVD following exposure to hypo-osmotic solution. The measurement of volume change was performed by acquiring an optical section from single cells loaded with the fluorescent dye calcein. Changes in intensity of fluorescence are proportional to changes in the concentration of the dye, which are strictly dependent on changes in cell water volume, allowing the measurement of relative changes in cell volume (6, 25). Unexpectedly, a significant proportion of both hCIC-3-GFP and the parental HEK293 cells, did not show RVD. The non-responsive cells readily converted calcine-AM to calcine, an indication of active esterases, and did not show leak of the dye indicating an intact plasma membrane. We do not have an explanation for the heterogeneity of response, although it was not CIC-3-dependent. The cells that did exhibit cell volume regulation showed no significant difference in RVD due to the expression of CIC-3 (Fig. 7B). Thus, CIC-3 does not appear to be involved in cell volume regulation.

hCIC-5 Is Not Activated by Cell Swelling—hCIC-5 is closely related to CIC-3, and a current associated with CIC-5 expression has been recorded previously in Xenopus oocytes and in transiently transfected HEK293 cells (19). We therefore used hCIC-5 as a control. Like the CIC-3-GFP cells, in the hCIC-5-GFP cell line a similar proportion of the fusion protein was

FIG. 2. Confocal images of cell lines expressing CIC-3-GFP and CIC-5-GFP fusion proteins. Three representative confocal optical sections (top, middle, and bottom) taken along the z axis of cells expressing short hCIC-3-GFP, short hCIC-3(N579K)-GFP, and hCIC-5-GFP. Green fluorescence corresponds to the GFP fusion proteins and red the propidium iodide staining of DNA. The majority of each of the GFP fusion proteins is localized to a large organelle close to the nucleus, presumably the Golgi, to small vesicles throughout the cytoplasm, and to the plasma membrane.

FIG. 3. Biotin labeling of plasma membrane GFP fusion proteins. Cells were reacted with biotin maleimide without (a) and with (b) permeabilization of the plasma membrane. The amount of sample loaded per lane corresponds to 200 μg of protein of the initial protein extract in panels a and 50 μg in panels b. Lane 1, short hCIC-3-GFP; lane 2, short hCIC-3(N579K)-GFP; lane 3, long hCIC-3-GFP; lane 4, hCIC-5-GFP. The top set of panels shows biotinylated proteins detected with an anti-GFP antibody, detecting the CIC-GFP fusion protein: The GFP fusion proteins are accessible to biotin maleimide before and after permeabilization. The bottom set of panels shows biotinylated proteins detected with an anti-SHC antibody: This intracellular protein is only biotinylated once the membrane is permeabilized demonstrating that the non-permeabilized cells were intact and that, therefore, the biotinylation of the CIC-GFP fusions reflects their insertion in the plasma membrane.
located in the plasma membrane (see above). However, in contrast to ClC-3-expressing cells, a typical strong outwardly rectifying current was measured in ClC-5-expressing cells in isotonic conditions (Fig. 8A), with an anion conductance at positive potentials: Cl \rightleftharpoons Br \rightleftharpoons I \rightleftharpoons F (Fig. 8C). This is similar to currents previously reported for ClC-5, and different in activation, rectification, and ion selectivity from the endogenous swelling-activated currents seen in parental HEK293 cells and in cells expressing hClC-3. Exposure of hClC-5-expressing cells to hypo-osmotic solution elicited a typical swelling-activated current indistinguishable in magnitude and anion selectivity from the endogenous currents of HEK293 control

FIG. 4. Swelling-activated chloride currents in ClC-3-expressing HEK293 cells. Representative chloride currents in iso-osmotic (top set of panels) and 7 min after exposure to 30% hypo-osmotic (bottom set of panels) solutions are shown. Cells were stimulated with square voltage pulses from −80 mV to +120 mV in 40-mV steps from a 0-mV holding potential. a, control HEK-293 cells; b, HEK293-short ClC-5-GFP; c, HEK293-long ClC-3-GFP; d, HEK293-short ClC-3(N597K)-GFP; e, HEK293-long ClC-3(N597K)-GFP; f, HEK293-short CLC-3 (no GFP fusion).

FIG. 5. Mean chloride currents in ClC-3 and ClC-5-expressing cells. The mean steady-state peak chloride currents for the indicated cell types at +120 mV are shown. a, the rates of chloride current activation were measured from 1 min prior to exposure to hypo-osmotic solution up to 10 min following hypo-osmotic solution and are plotted as a fraction of the maximal steady-state currents at +120 mV. The data were fitted with a sigmoidal function. No significant difference was observed between the rates of activation of any of the cell lines generated. The data shown here are representative of only two cell lines, expressing short ClC3-GFP and ClC-5-GFP, for clarity. b and c, the mean currents in iso-osmotic (panel b, black bars) and after 7 min of exposure to 30% hypo-osmotic solution (steady state) (panel c, open bars) are shown. In panel c the open circles show the individual current readings obtained for different cells, demonstrating the heterogeneity between individual cells. Note the differences in pA/pF scales between b and c. No significant difference in currents between cell types were observed except for ClC-5 expressing cells under iso-osmotic conditions (**). These currents showed characteristics of previously described ClC-5 currents (see also Fig. 8). The number of cells recorded for each cell line (n) were: HEK293, n = 9; short hClC-3-GFP, n = 9; long hClC-3-GFP, n = 5; short N579K hClC-3-GFP, n = 5; short hClC-3, n = 3; hClC-5-GFP, n = 9.

FIG. 6. Anion permeability sequences for cells expressing hClC-3-GFP variants. Cells were stimulated with a ramp protocol from −80 mV to +80 mV lasting 1 s in the presence of different halides as indicated.

FIG. 7. RVD is unaffected by expression of ClC-3. Cell volume recovery (RVD) following exposure to a 40% hypo-osmotic solution was measured. A, a representative time course for relative change in cell volume (Vt/Vo) for an HEK293 cell. B, mean values for %RVD calculated from cell volume changes in single cells after 5-min exposure to hypo-osmotic conditions. Control HEK293 cells, n = 22; ClC-3-GFP-expressing cells, n = 15. No significant difference was measured.
cells or hClC-3-GFP-expressing cells (Fig. 8, B and D). These data demonstrate that, unlike hClC-3, hClC-5 is constitutively active under the conditions used and elicits characteristic currents very different from the cell swelling-activated currents seen in HEK293 cells. These data provide a positive control for the absence of currents seen when expressing ClC-3 and also demonstrate that addition of a GFP tag to ClC-5 has no significant effect on current magnitude or characteristics.

Changes in Intracellular Calcium Do Not Activate hClC-3—The data above provide strong evidence against the hypothesis that ClC-3 is responsible for cell swelling-activated chloride currents, and also show that (unlike ClC-5) if ClC-3 is a chloride channel at all, it is not constitutively active under the recording conditions used in this study. The other chloride channels that have been characterized in epithelial cells are activated by change in intracellular Ca$^{2+}$. We therefore studied cells expressing hClC-3 in response to elevations of intracellular Ca$^{2+}$ using the calcium ionophore A23187 (Fig. 9B). This stimulus elicited, in both ClC-3-expressing HEK293 and untransfected HEK293 cells (Fig. 9A), currents with properties similar to well-characterized epithelial Ca$^{2+}$-activated chloride channels (31–33). These currents are easily distinguishable in kinetic characteristics from the cell-swelling-activated current. Thus, hClC-3 does not appear to encode a calcio-activated chloride channel.

Tissue Distribution of ClC-3—Regulatory volume decrease is a general property of almost all cell types (2, 34). Similarly, a typical I$_{\text{Cl, Swell}}$ has been identified in most cell types studied (4, 5). Therefore, any candidate protein for I$_{\text{Cl, Swell}}$ might be expected to have a widespread tissue distribution. We examined expression of ClC-3 by Western blotting in a wide array of murine tissues using an antibody against the N terminus of ClC-3 (D1). Although the D1 antibody, like the Alomone antibody, is not absolutely specific for ClC-3, comparison of the two antibodies allowed us to unambiguously identify protein bands that correspond to ClC-3. It is important to note that ClC-3 runs as multiple bands in SDS gels. This may reflect the predicted long and short versions, because D1 recognizes both versions (Fig. 1), or may reflect different post-translation modifications. Very high levels of ClC-3 were detected in kidney and the central nervous system. However, for all other tissues examined (thymus, lung, liver and spleen, heart, skeletal muscle, upper and lower intestine, and testis) little or no signal was detected (Fig. 10A). The absence from heart is in contrast to a previous report (22) (Fig. 10B, right panel). To resolve this apparent discrepancy we also probed the blot with Alomone anti-ClC-3 antibody. This antibody (Alomone) did detect a band in cardiac tissue (Fig. 10B, left panel), but because this was not detected by antibody D1 it must reflect a lack of specificity of the Alomone antibody rather than the presence of ClC-3 in the tissue. In conclusion, the distribution of ClC-3 is consistent with a specific role of ClC-3 in kidney and central nervous system, rather than with a more general role in RVD.

DISCUSSION

Cell volume regulation is an important property of all cells, and characteristic cell swelling-activated chloride currents...
play an important role in RVD in most cell types. Despite intensive study, the molecular identity of the channel(s) underlying $I_{\text{Cl}, \text{Swell}}$ has been elusive. Recently, CIC-3 has been proposed as a candidate for $I_{\text{Cl}, \text{Swell}}$ with a role in cell volume regulation (1, 17, 18, 22). However, this conclusion has been questioned (19) and further complicated by the identification of two potential isoforms, a short and long version of CIC-3 (20). In this study we have rigorously assessed whether CIC-3 is responsible for $I_{\text{Cl}, \text{Swell}}$ and conclude that CIC-3 is neither responsible for $I_{\text{Cl}, \text{Swell}}$ nor plays a role in RVD.

Cell lines overexpressing hCIC-3 were generated. The use of GFP fusions and extracellular biotinylation were used to demonstrate that a significant proportion of CIC-3 was inserted in the plasma membrane. Neither the short nor long form of CIC-3, when overexpressed at the surface of HEK293 cells, had any significant influence on $I_{\text{Cl}, \text{Swell}}$. Thus, unless a very large number of cells is studied in a controlled and rigorous fashion, and no selection of these “background” currents can vary substantially from cell to cell, and, perhaps more importantly, their magnitude is sensitive to clarification of tissue distribution. It appears that CIC-3 is relatively restricted in its distribution, being expressed primarily to the opposite selectivity ($I^- > Cl^-$). We observe no such currents generated by either the long or short version. Thus, the conditions or accessory proteins required to activate CIC-3 are unknown. Either way, these results are incompatible with CIC-3 being responsible for known chloride currents at the plasma membrane and are consistent with an intracellular role.

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Addendum—When this article was under review Stobrawa et al. (36) showed that disruption of the Clcn3 gene does not impair swelling-activated currents.

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