CIC-2 Contributes to Native Chloride Secretion by a Human Intestinal Cell Line, Caco-2*

Received for publication, July 27, 2000, and in revised form, November 26, 2000
Published, JBC Papers in Press, November 28, 2000, DOI 10.1074/jbc.M006764200

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It has been previously determined that CIC-2, a member of the CIC chloride channel superfamily, is expressed in certain epithelial tissues. These findings fueled speculation that CIC-2 can compensate for impaired chloride transport in epithelial tissues affected by cystic fibrosis and lacking the cystic fibrosis transmembrane conductance regulator. However, direct evidence linking CIC-2 channel expression to epithelial chloride secretion was lacking. In the present studies, we show that CIC-2 transcripts and protein are present endogenously in the Caco-2 cell line, a cell line that models the human small intestine. Using an antisense strategy we show that CIC-2 contributes to native chloride currents in Caco-2 cells measured by patch clamp electrophysiology. Antisense CIC-2-transfected monolayers of Caco-2 cells exhibited less chloride secretion (monitored as iodide efflux) than did mock transfected monolayers, providing the first direct molecular evidence that CIC-2 can contribute to chloride secretion by the human intestinal epithelium. Further, examination of CIC-2 localization by confocal microscopy revealed that CIC-2 contributes to secretion from a unique location in this epithelium, from the apical aspect of the tight junction complex. Hence, these studies provide the necessary rationale for considering CIC-2 as a possible therapeutic target for diseases affecting intestinal chloride secretion such as cystic fibrosis.

The physiological significance of CIC-2, a ubiquitously expressed member of the CIC family of chloride channels (1) is not fully understood. Based primarily on studies of native CIC-2 message and protein expression, roles for CIC-2 in neuronal and epithelial tissue have been proposed (2, 3). ClC-2 channel activity has been implicated in the regulation of neuronal responses to GABA-A receptor interaction (2). In non-neuronal cells, CIC-2 function has been linked to volume regulation, and in epithelial cells, it has been linked specifically to chloride secretion (3).

* This work was funded by a National Institutes of Health grant (to C. E. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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Immunolocalization studies of CIC-2 revealed that it is situated on the apical surface of airway epithelial cells in neonatal rat airways (4). Subsequent Ussing chamber studies showed that luminal acidity promoted chloride secretion in neonatal airway cells via a cadmium-sensitive channel (5). These findings prompted us to suggest that CIC-2, a channel that exhibits these properties, may mediate chloride secretion in neonatal rat airways. In addition, Schwiebert et al. (6) reported that CIC-2-like currents are present in airway epithelial cells derived from an adult patient with cystic fibrosis (CF) and suggested chloride transport via CIC-2 may be able to compensate for defective or absent CFTR chloride channels in the CF airway epithelium. Further, because CIC-2 message can be detected in intestinal epithelial tissue obtained from cftr-knockout mice, Joo et al. (7) also suggested that there is the potential for CIC-2 to provide a bypass pathway for chloride transport in CF affected intestines. Despite these intriguing observations, there was no direct molecular evidence to suggest that CIC-2 contributes to native chloride secretion.

In the present work, we assessed the role of CIC-2 in chloride secretion in the Caco-2 cell line, a cell line that models the human small intestinal epithelium (8, 9). Using immunofluorescence and confocal microscopy, we confirmed that CIC-2 protein is endogenously expressed in the plasma membrane of Caco-2 cells. Further, we show that it is uniquely situated at the apical aspect of the tight junctions between cells in fully differentiated monolayers of these cells. Using an antisense strategy, we show that endogenously expressed CIC-2 mediates currents across the plasma membrane of single Caco-2 cells and, finally, that CIC-2 can contribute to native anion secretion across Caco-2 cell monolayers.

EXPERIMENTAL PROCEDURES

Caco-2 Cell Culture—Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). They were grown in Earl’s minimum essential medium (Wisent Inc.) containing 10% fetal calf serum, with 2 mM glutamine, 100 units penicillin G, and 100 μg/ml streptomycin sulfate at 37 °C in an atmosphere of 5% CO₂, 95% air. For patch clamp studies, cells were used 1–2 days after plating onto 35-mm coverslips (Fisher). For Ussing chamber studies of chloride currents and assessment of CIC-2 localization by confocal microscopy, Caco-2 cells were seeded at high density (1 × 10⁶ cells/ml⁻¹, 500 μl/filter) and grown to confluency on clear Snapwell (Costar) filters (pore size, 4 μm; diameter, 12 mm). Filters were cultured at 37 °C in an atmosphere of 5% CO₂, 95% air, for 2 weeks with medium replacement every 2–3 days. The formation of an intact monolayer was assessed by measuring transepithelial resistance in Ussing chambers. Transepithelial resistance was calculated using Ohm’s law, from measurements of the change in
short circuit current measured (Isc, μA) upon passing 1 mV across the epithelium. Monolayers were considered acceptable when the transepithelial resistance exceeded 500 Ohms/cm² and the transepithelial potential difference exceeded 2 mV. Alternatively, for iodide efflux studies and assessment of protein expression by Western blotting and confocal microscopy, monolayers were infected with the adenovirus (45) to facilitate ClC-3 detection. The bath solution contained 140 mM NaCl and incubated with 900 mM formaldehyde and transferred to Hybond-N membranes (Amer sham Pharmacia Biotech). Blots were cross-linked with UV radiation and hybridized with mouse-specific ClC-2 DNA fragments radiolabeled by random priming (10). Final conditions of washing included 0.2 × SSC (sodium chloride/sodium citrate) with 0.1% SDS at 60 °C. The blots were exposed to X-Omat film (Kodak) for 24–72 h at −70 °C with one intensifying screen.

Western Analysis—Caco-2 cells, grown on 60-mm plastic dishes, were washed with phosphate-buffered saline containing 10 mM monobasic mix, pH 6.8, and 150 mM NaCl, final pH 7.2, and incubated with 900 μl of lysis buffer containing 1% Triton X-100, 120 mM NaCl, 10 mM Tris, 25 mM KCl, 25 mM MgCl₂, 1.8 mM CaCl₂, and protease inhibitors leupeptin (10 μg/ml) and aprotinin (10 μg/ml), 1 mM benzamidine, 0.5 mM E64, and 2 mM PMSF. The cells were washed with the lysis buffer and sonicated for 1 min and vortexed. The supernatant was then centrifuged for 20 min at 4 °C at 80,000 × g to isolate a crude membrane preparation. Following protein assay of the supernatant, 50 μg of this preparation was analyzed by SDS-polyacrylamide gel electrophoresis (8% gel) using anti-CIC-2 antibody at a concentration of 2 μg/ml. This polyclonal antibody, as described previously, was generated against a GST fusion peptide containing amino acids 31–74 of rat ClC-2 (rClC-2 cDNA kindly provided by T. Jentsch). The CIC-2-specific antibody was immunopurified from a matrix of GST-N-peptide coupled on an activated agarose column as previously described (11, 12). The monoclonal antibody against β-actin (Sigma, anti-β-actin clone AC-74) was used at 1/1000 dilution. Immunoreactive protein was detected using the ECL system (Amer sham Pharmacia Biotech).

Immunofluorescence—Immunofluorescence labeling was performed on Caco-2 cells grown on 35-mm circular coverslips or on clear 35-mm, 0.4-μm pore Snapwell (Corning Costar) filters. The pattern of CIC-2 labeling was identical regardless of the support employed. Cells were fixed with paraformaldehyde AM (4% in phosphate-buffered saline) and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline. Cells were incubated for 0.5 h at 25 °C in 0.5% Triton X-100 in Triton-X100-buffered saline containing 10 mM Tris-Cl and 150 mM NaCl with final pH 8, and then for 2.5 h with the polyclonal antibody against CIC-2 (30.7 μg/ml) or overnight in the refrigerator with the polyclonal antibody against CIC-3 (30 μg/ml) (Alomone Labs Ltd., Jerusalem, Israel). Then the cells were washed and incubated with Texas Red-conjugated or fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (0.02 mg/ml; Molecular Probes) and washed again before mounting. For colocalization studies of CIC-2 and the tight junction protein occludin, the above procedure was followed by additional incubation with the monoclonal anti-occludin antibody (0.002 mg/ml, Zymed Laboratories Inc., Mississauga, Canada) for 1 h and washed. Cells were then incubated with Texas Red-conjugated anti-mouse secondary antibody (0.02 mg/ml; Molecular Probes) and washed before mounting. For the competition studies, the anti-CIC-2 antibody was preincubated with 2-fold excess of the antigenic fusion peptide overnight at 4 °C before incubation. Slides were viewed on an Olympus Vanox AHB3 microscope using epifluorescence, and images were captured using the Image Pro Plus program (Cybernetics, L.P.). For confocal microscopy, sections (each 0.7 μm in thickness) were viewed with a 10× objective on a Leica TCS 4D microscope, and the images were captured using the SCANware 5.01 program.

Patch Clamp Studies of Caco-2 Cells—Caco-2 cell membrane currents were measured using conventional whole cell patch clamp technique (13). Patch clamp electrodes were prepared from borosilicate glass capillaries (outer diameter, 1.5 mm; inner diameter, 1.18 mm) with an inner filament (World Precision Instruments, Inc., Sarasota, FL). Typically, 12–15 μl of PP-85 patch pipet electrode was used for the standard two-pull technique. The tip resistance was 3–5 MΩ when filled with pipette solution (see below for composition). Whole cell currents were measured using an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA) and were filtered at 100 Hz with a 6-pore Bessel Filter. Sampling rate was 4 kHz for most data, and junction potentials were corrected. Voltage clamp protocols were generated using pCLAMP software (version 7, Axon Instruments) via a Pentium II computer interfaced with a 1200 series Digidata (Axon Instruments) The same software package was used both for data acquisition and analysis. Current-voltage relationships were determined in a stepwise clamp protocol from a holding potential of −30 mV, voltage pulses of 5.0 mV from −160 mV to −50 mV in 20 mV increments were applied, and the prepulse was applied for 100 ms. The bath on glass coverslips to achieve a differentiated phenotype, as documented by Sood et al. (9).
vector alone as described under "Experimental Procedures." Only monolayers possessing 1 × 10^6 cells after the entire transfection protocol were used for subsequent assays. The transfected cells were iodide loaded according to established methods (14, 15) using a 1-ml Ringers Nitrate loading buffer at pH 7.4 containing 136 mM NaI, 4 mM KNO_3, 2 mM Ca(NO_3)_2, 2 mM Mg(NO_3)_2, 11 mM glucose, and 20 mM HEPES. The cells were incubated for 1 h at 37°C in the presence of 5% CO_2 in the above buffer. After this incubation period, the coverslips were fixed with cold 4% paraformaldehyde × 2 for 15 min at 4°C. After washing, the coverslips were incubated sequentially with 10% normal goat serum, 2% BSA, 0.01% Tween-20 PBS containing the polyclonal anti-ClC-2 antibody (pCLAMP 6.04, Axon Inst.) and the antigenic fusion protein (ppt, second lane) but not with GST alone (third lane), confirming antibody specificity.

**A**

**B**

**FIG. 1. CIC-2 message and protein expression in Caco-2 cells.** A, Northern analysis shows that the CIC-2 cDNA probe recognizes a band of ~4.6 kb in Caco-2 cells. The 6.2-kb marker is CFTR, and the 4.6- and 1.8-kb markers indicate the position of ribosomal RNA. B, immunoblot analysis shows that the polyclonal anti-CIC-2 antibody generated against a peptide within the amino terminus of the rat ClC-2 sequence (residues 34–71) (11) recognizes a broad band that corresponds to molecular mass of ~97 kDa in Caco-2 cells (first lane). This 97-kDa band is competed with 1.2-fold excess of the antigenic fusion peptide (ppt, second lane) but not with GST alone (third lane), confirming antibody specificity.

**FIG. 2. Immunolocalization of CIC-2 in the plasma membrane of Caco-2 cells.** A, image of CIC-2 localization in Caco-2 cell membranes by immunofluorescence using an epifluorescence microscope (see "Experimental Procedures"). B, the immunofluorescence specifically labels CIC-2 as it can be competed by preincubation with 1.9-fold excess of the CIC-2 fusion protein (ppt, right panel). Images were obtained using a 40× objective.

Endogenous ClC-2 Can Contribute to Cl⁻ Secretion

CIC-2 mRNA in Caco-2 cells was detected by Northern blot analysis as a 4.6-kb transcript (Fig. 1A). This size transcript plus a smaller transcript of ~3.3 kb in size has been detected in several other tissues and cell lines, as well, including the colonic epithelial cell line T_84 (6). Immunoblot analysis (Fig. 1B) using a polyclonal antibody directed against CIC-2 (12) showed that CIC-2, migrating as a 90–97-kDa protein, is expressed in Caco-2 cells. This signal was competed continuously after placing the coverslip into an isotonic solution (the above Ringers Nitrate efflux buffer) or hypotonic solutions (osmolarity adjusted to 300 mOsm with added sucrose. After this washing period, efflux of cellular iodide was assessed continuously after placing the coverslip into an hypotonic solution (a 1 ml solution containing 136 mM NaNO_3, 4 mM KNO_3, 2 mM Ca(NO_3)_2, 2 mM Mg(NO_3)_2, 11 mM glucose, and 20 mM HEPES. The cells were incubated for 1 h at 37°C in the presence of 5% CO_2 in the above buffer. After this incubation period, the coverslips were fixed with cold 4% paraformaldehyde × 2 for 15 min at 4°C. After washing, the coverslips were incubated sequentially with 10% normal goat serum, 2% BSA, 0.01% Tween-20 PBS containing the polyclonal anti-ClC-2 antibody (pCLAMP 6.04, Axon Inst.) and the antigenic fusion protein (ppt, second lane) but not with GST alone (third lane), confirming antibody specificity.

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currents was less inwardly rectifying than that observed in vector-alone transfected cells. We verified by assessing antisense ClC-2 transfected Caco-2 cells relative to control that there is a 70% decrease in ClC-2 protein quantity in transfected Caco-2 cells. Using the NIH Imaging Program, we found Western analysis of cell lysates from ClC-2 antisense-transfection of ClC-2 antisense cDNA (see “Experimental Procedures”). The currents measured in isotonic condition (−32 ± 2 pA/pF) (p = 0.0208). The I/V relationship of the HTS-stimulated chloride currents was less inwardly rectifying than that observed in isotonic solutions (Fig. 3C). A similar change in the I/V relationship was observed with HTS in chloride currents specifically conferred by ClC-2 expression in Xenopus oocytes and Sf9 cells and has been attributed to an alteration in the inactivation gate of ClC-2 (11, 16). These results indicate that native ClC-2 expression at the cell surface of Caco-2 cells is associated with appearance of chloride currents with activation and conductance properties similar to those conferred by ClC-2 expression in heterologous expression systems (1, 11, 16, 18).

We used an antisense strategy to confirm that the above currents were mediated by ClC-2 because the pharmacological approach lacks specificity. First, we confirmed that transient transfection of ClC-2 antisense cDNA (see “Experimental Procedures”) successfully reduced ClC-2 protein expression by Western analysis of cell lysates from ClC-2 antisense-transfected Caco-2 cells. Using the NIH Imaging Program, we found that there is a 70% decrease in ClC-2 protein quantity in antisense ClC-2 transfected Caco-2 cells relative to control (vector-alone transfected cells). We verified by assessing β-actin expression that differences in protein loading could not account for the decrease in ClC-2 expression in the antisense transfected cells (Fig. 4A). Furthermore, we examined the effects of antisense ClC-2 transfection on immunolabeled Clic-2 detected by fluorescence confocal microscopy. DNA coding for green fluorescence protein (GFP) was cotransfected with antisense ClC-2 (or empty vector as a control) into Caco-2 cells to identify transfected cells (Fig. 4B). We used an imaging program (Scion Corp.) to compare the ClC-2 immunofluorescence intensity in antisense ClC-2 and in vector transfected Caco-2 cells. We found that the fluorescence intensity of the signal (red) corresponding to membrane expression of ClC-2 was reduced by ~75% in antisense ClC-2 transfected Caco-2 cells (24.9 units ± 4.3, n = 13, p < 0.0001) relative to the intensity of the ClC-2 signal in mock transfected cells (105.8 units ± 3, n = 10). Immunofluorescence corresponding to expression of ClC-3, a related family member, was not affected by antisense ClC-2 transfection (Fig. 4C). The signal detected using this ClC-3 antibody in immunofluorescence studies can be competed using the antigenic peptide used to raise the antibody, confirming its specificity (19). Fig. 4C shows that the ClC-3 immunofluorescence (red) in antisense ClC-2 and GFP cotransfected Caco-2 cells (106.1 units ± 3.2, n = 20) was similar to that in vector and GFP cotransfected cells (105.9 units ± 2.2, n = 17, p = 0.97). Interestingly, our studies show that unlike ClC-2, immunoreactive ClC-3 appears to be primarily expressed in intracellular membranes, although there is signal detected at the cell surface in a subpopulation of cells.

For patch clamp studies, we manipulated Clic-2 expression using intranuclear plasmid injection technique (20, 21), because this method permits control of plasmid copy number and hence has greater precision in manipulating the level of antisense expression. Fluorescein isothiocyanate-dextran was co-injected with the plasmid to permit identification of manipulated cells. We found that microinjection of antisense ClC-2 cDNA into Caco-2 cells decreased the ClC-2-like currents in a dose-dependent manner (Fig. 5, A and B). The negative whole cell current measured at −160 mV decreased from −37 ± 1 pA/pF (n = 8) in uninjected cells to −25 ± 2 pA/pF (n = 5, p = 0.0003) and −12 ± 1 pA/pF (n = 10, p < 0.0001) in 50 and 300 μg/ml antisense ClC-2 cDNA injected cells, respectively (Fig. 5, A and B). To allow direct comparison of current amplitude in ClC-2 antisense injected and uninjected Caco-2 cells, we normalized these currents to the currents at −160 mV in uninjected cells (Fig. 5B). As shown in Fig. 5B, the normalized ClC-2 currents in cells injected with 50 or 300 μg/ml antisense plasmid were decreased by 29 ± 6 and 68 ± 2%, respectively.

Inhibition of hyperpolarization-activated chloride currents by Clic-2 antisense expression was a specific response, because nuclear injection of antisense ClC-4, a distinct membrane of the CIC chloride channel family (3), did not affect these endogenous currents (Fig. 5C). The currents measured in antisense ClC-4 injected Caco-2 cells (−31 ± 3 pA/pF at −160 mV, n = 4) was not significantly different from that measured in noninjected cells (−35 ± 2 pA/pF, p = 0.1849). Fig. 5D shows that the amplitude of the hyperpolarization-activated, inwardly rectifying chloride current was doubled by expression of exogenous ClC-2 cDNA (−66 ± 3 pA/pF, n = 4). Together, these results indicate that CIC-2 natively expressed in Caco-2 cells mediates inwardly rectifying, hyperpolarization-activated chloride currents.

To determine the contribution of CIC-2 to HTS-stimulated chloride currents in Caco-2 cells, we studied the effects of antisense ClC-2 transfection on the development of hypotonicity-activated chloride currents in Caco-2 cells. As shown in Fig. 5E, the amplitude of the HTS-stimulated chloride currents...
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Fig. 4. ClC-2 antisense reduces ClC-2 protein expression in Caco-2 cells. A. Western analyses show reduced ClC-2 expression in ClC-2 antisense transfected monolayers of Caco-2 cells. 50 µg of protein were loaded per lane. β-actin labeling confirms that the reduction of ClC-2 signal does not reflect less sample. B. Upper panels show confocal image of immunolabeled ClC-3 (red) endogenously expressed in Caco-2 cell cotransfected with cDNA coding for empty vector (Vtr) and GFP. Lower panels, the immunofluorescence corresponding to ClC-2 signal in antisense ClC-2 and GFP cotransfected Caco-2 cells. Images were obtained using a 65x objective. Fluorescence intensity of the ClC-2 signal was quantitated by averaging the pixel intensity of the grayscale image of immunofluorescence corresponding to ClC-2 signal in antisense ClC-2 and GFP cotransfected Caco-2 cells. Images were obtained using a 63x objective. Fluorescence intensity of the ClC-2 signal was quantitated by averaging the pixel intensity of the grayscale image of eight randomly selected regions around the nucleus delimited using four lines transecting the nucleus. The bar graph shows the means ± S.E. of fluorescence intensities corresponding to ClC-2 membrane expression for vector (n = 10) and antisense ClC-2 transfected (aClC-2, n = 13) Caco-2 cells. C, the upper panels show confocal image of immunolabeled ClC-3 (red) endogenously expressed in Caco-2 cell cotransfected with cDNA coding for empty vector and GFP. Lower panels, the immunofluorescence corresponding to ClC-3 signal in antisense ClC-2 and GFP cotransfected Caco-2 cells. Fluorescence intensity of the ClC-3 signal was quantitated by averaging pixel intensity of the grayscale image of eight randomly selected regions around the nucleus delimited using four lines transecting the nucleus. The bar graph shows the means ± S.E. of fluorescence intensity determined in vector (n = 17) and antisense ClC-2 transfected (n = 20) Caco-2 cells.

Fig. 5. ClC-2 antisense reduces hyperpolarization and hypotonic activated chloride currents in Caco-2 cells. A. Hyperpolarization activated chloride currents are reduced in Caco-2 cells microinjected (intranuclear) with antisense ClC-2 plasmid (300 µg/ml). Whole cell currents obtained by voltage steps of 20 mV increments, applied from −160 mV to +40 mV. Initial holding potential, −30 mV; final holding potential, −60 mV. The patch pipette and bath solutions both contained NMGND chloride solutions (see “Experimental Procedures” for more detail on buffers). B. Mean current-voltage relationship for chloride currents obtained from control Caco-2 cells (n = 8, Ctrl) from cells injected with 50 µg/ml ClC-2 antisense (n = 5, aClC-2) and 300 µg/ml ClC-2 antisense (n = 10). Currents were normalized to cell capacitance (pF). The bar graph shows mean currents in control and ClC-2 antisense microinjected Caco-2 cells at −160 mV. Currents were normalized to the mean current measured in un.injected cells at −160 mV. C, mean IV curves of hyperpolarization-activated chloride currents in antisense ClC-2 injected (square, n = 4) and in noninjected (circle, n = 4) Caco-2 cells. C. Mean IV curves of hyperpolarization-activated chloride currents at −160 mV (square, n = 7 and after (Hypo, circle, n = 7)) Caco-2 cells. Antisense ClC-2 expression significantly diminished chloride currents measured at −160 mV and +40 mV (p < 0.0001; *, p = 0.0053). F, mean IV curves of ClC-2 current under hypotonic condition in antisense ClC-2 injected (square, n = 7) and in noninjected (circle, n = 4) Caco-2 cells. Antisense ClC-2 expression significantly diminished chloride currents measured at −160 mV and +40 mV (p = 0.006). In a Polarized Caco-2 Cell Monolayer, CIC-2 Localizes Close to the Tight Junction Complex—To determine whether ClC-2 protein exhibits a polarized distribution in fully differentiated intestinal cells, we examined its subcellular localization in confluent Caco-2 cells grown on semipermeable filters. It has been well documented that Caco-2 cells grown on either filters or on glass exhibit a polarized phenotype 4–6 days after plating (8, 9). In such a confluent monolayer, we found that ClC-2 protein exhibited a novel localization pattern. Fig. 6 shows optical sections obtained using a confocal microscopy of confluent Caco-2 cells co-labeled with the polyclonal...
anti-CIC-2 antibody and a monoclonal antibody against the transmembrane tight junction protein, occludin (22). The first row of images shows the most apical sections, and the subsequent rows show consecutive images obtained toward the basolateral pole. The CIC-2 signal (red) clearly delineates the plasma membrane in the first row (Fig. 6), whereas the occludin signal (green) is quite faint, suggesting that CIC-2 protein expression is apical relative to this tight junction protein. The yellow signal, indicative of regions of overlap, is weak in this apical section. In the next row of images, the membrane staining corresponding to both proteins and the yellow signal is intense, suggesting that expression of these proteins may overlap in this optical section. The last two rows of sections show the disappearance of the CIC-2 signal from the plasma membrane while the occludin signal remains strong (Fig. 6). Thus, it appears that CIC-2 localization overlaps with the apical aspect of the tight junctions. Identical localization of CIC-2 was observed for confluent monolayers of Caco-2 cells grown on glass coverslips (data not shown).

CIC-2 Contributes to Hypotonicity-stimulated Chloride Secretion in Monolayers of Caco-2 Cells—There may be multiple functional consequences of this unique localization of CIC-2 because the tight junction is known to be critical for regulating solute (including ions) and water flux through the paracellular pathway and as well maintaining the polarization of membrane proteins and lipids (22, 23). We reasoned that CIC-2 may contribute to chloride flux in a secretory direction across the apical membrane because its localization not only overlaps with the junction transmembrane protein, occludin, but it also extends into the membrane at the apical aspect of the tight junction. Typically, chloride channels implicated in secretion have been localized to the apical membrane (24). Hence, we investigated the role of CIC-2 in chloride secretion across monolayers of Caco-2 cells.

The application of hypotonicity to the mucosal or apical surface of intestinal epithelia has been previously shown to stimulate chloride secretion (25, 26). However, the molecular basis for this secretory response remained unclear. To determine whether Caco-2 cell monolayers model this response, we measured short circuit current responses to hypotonic shock by Caco-2 cell monolayers mounted in Ussing chambers. Hypotonicity-activated increases in luminally (or apically) directed negative short circuit current \( I_{sc} \) across epithelia have been shown to correlate with chloride secretion in previous studies (25, 26). Similarly, we found that reducing the osmolarity of the bath facing the apical membrane of confluent Caco-2 cells (by 20%) evoked a transient increase in luminally directed, negative transepithelial short circuit current \( I_{sc} \) (Fig. 7A). \( I_{sc} \) increased significantly from \(-1.0 \pm 0.1 \mu A/cm^2\) to \(-3.1 \pm 0.4 \mu A/cm^2\) \((n = 14, p < 0.0001, \text{Student's test for paired data})\) with this treatment. These findings are consistent with the transient activation of an apical chloride conductance path by luminal hypotonicity in Caco-2 cells.

To determine directly whether CIC-2 contributes to this response, we assessed the effect of antisense CIC-2 transfection. The above Ussing chamber studies required that Caco-2 cell monolayers were grown on semipermeable filter supports. We found that transfection of Caco-2 cells on such filters was not efficient compared with transfection of monolayers on coverslips (see Western analysis on Fig. 4). Hence, we utilized an iodide efflux method to monitor chloride secretion from cells on glass coverslips. This method has been used extensively in the study of chloride secretion through CFTR in epithelial monolayers (14, 15). In Fig. 7B, we show traces of the time-dependent efflux of iodide from iodide loaded Caco-2 cell monolayers. Efflux from monolayers (each comprised of \(1 \times 10^6\) cells) into control, iodide-free isotonic solutions reflects efflux through constitutively open anion channels. The rate of iodide efflux is enhanced if monolayers are transferred to hypotonic (iodide-free) solutions (25% isotonicity) as shown in the traces in Fig. 7B, presumably because of the activation of apically localized chloride channels. The extent of iodide efflux measured during the first minute has been plotted in the bar graph shown in Fig. 7B (right panel). We show that in mock (vector-alone) transfected Caco-2 cell monolayers, the rate of iodide efflux is much greater in monolayers exposed to hypotonic solutions relative to the efflux rate from monolayers exposed to isotonic solutions \((p = 0.002)\). In antisense CIC-2 transfected monolayers, the rate of iodide efflux in hypotonic solutions was significantly reduced in comparison to the hypotonicity-evoked efflux measured in monolayers transfected with vector alone \((p = 0.001)\). This result suggests that CIC-2 contributes to hypotonicity-activated anion secretion. On the other hand, overexpression of CIC-2 does not affect the iodide efflux response in hypotonic

Fig. 6. Localization of CIC-2 protein to the apical aspect of the tight junctions formed between Caco-2 cells in a confluent monolayer. Consecutive optical cross-sections of confluent Caco-2 cells that have been co-labeled with the polyclonal anti-CIC-2 antibody (red) and a monoclonal anti-occludin antibody (green) were obtained by confocal microscopy. Panels 1–5 extend from the apical toward the basolateral membrane. Panel 2 is the most apical section in which the CIC-2 signal could be detected, and panel 5 is the last section in which membrane staining of CIC-2 could be detected. Pictures were taken using a confocal microscope with a 100× objective.
short circuit current response to dilution of mucosal solution. A tran-
on semipermeable support were mounted in Ussing chamber to assess
by hypotonic shock (

ers were studied for each condition.

represent data from hypotonic solutions. A minimum of four monolay-

bars

as monolayers transfected with ClC-2 in the sense orientation. The

fected and antisense ClC-2 transfected Caco-2 cell monolayers as well

channels contributing to this hypotonicity-activated

DISCUSSION

The major aim of this study was to test the hypothesis that

solutions (p = 0.33) but does appear to increase the basal efflux

(p = 0.009). Hence, overexpression of CIC-2 confers an increase

in basal anion secretion. Hypotonic solutions do not cause a

further increase in iodide efflux in cells overexpressing ClC-2,

possibly because the high basal permeation rate quickly dissi-

pates the driving force for further iodide flux.

solutions that act to increase potassium permeability, i.e. acetyl-

choline and vasoactive intestinal peptide (26, 30). The acetyl-

choline analogue, carbachol, has been reported to cause

transient hyperpolarizations of 10–25 mV in isolated small

intestinal crypts. Hence, during stimulation with the above

hormones, CIC-2 channels may become further activated. In

the present studies we diluted the external solutions to 70–

80% isotonicity to observe stimulation of CIC-2-mediated cur-

rents in single cells and iodide efflux from Caco-2 cell mono-

layers. It remains to be determined whether this experimental

maneuver reflects a physiologically relevant stimulus to intes-

tinal epithelial cells such as the generation of osmotic gradients

during the concentrative uptake of nutrients (31).

Several previous studies have suggested that luminal hypo-

tonicity induces transepithelial chloride secretion. A study on

Necturus enterocytes has shown that a swelling-activated chlo-

ride conductance is present in the apical membrane of these

cells (32). In addition, cell swelling may stimulate transepithelial

circle secretion in airway epithelium (33), the T84 colonic

epithelial cell line (34, 35), HT-29Cl.19A intestinal epithelial

cells (36), and rat ileum (25). The molecular identity of the

chloride channels contributing to this hypotonicity-activated

secretory chloride conductance in these diverse epithelial tis-

sues has not been determined. Our current studies showing

that antisense CIC-2 transfection reduced this function sug-

gests that CIC-2 should be considered as a candidate. However,

we cannot rule out the possibility that antisense CIC-2 trans-

fection may have caused primary and/or secondary changes in

the expression of other proteins over the 48-h transfection time

period. It is conceivable that expression of undefined paralogs

of human CIC-2 may have been reduced directly by the anti-

sense rodent CIC-2 construct employed in the current studies.

Further, although the expression of another channel impli-

R. Gyo¨mo¨rey and C. Bear, unpublished data.

Similarly, CIC-2 protein has been localized to the apical mem-

brane of rat neonatal respiratory epithelium (4, 5) and may

mediate secretion in this tissue. However, CIC-2 is not likely

to contribute to chloride secretion in all transport epithelia.

Preliminary studies in our laboratory have localized CIC-2 to the

basolateral membrane of the mouse colon; hence, CIC-2 protein

is likely to mediate chloride reabsorption by this tissue.

The mechanisms for activation of CIC-2 channel function in situ

are currently unclear. In neurons, CIC-2 is basally active at

resting membrane potentials (2). The results of the present

experiments suggest that in epithelial cells, CIC-2 channels are

partially active at resting membrane potentials. Patch clamp

studies of Caco-2 cells revealed that chloride currents associ-

ated with CIC-2 expression, i.e. those endogenous currents

inhibited by CIC-2 antisense and augmented by expression of

exogenous CIC-2 expression, are activated by membrane hyper-

polarization to potentials more negative than −60 mV. This

membrane potential is close to the resting membrane potential

cited for gastrointestinal epithelial cells, i.e. from −50 to −60

mV (26). Gastrointestinal epithelial cells can, however, reach

more hyperpolarized potentials when stimulated by the hor-

mones that act to increase potassium permeability, i.e. acetyl-

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Endogenous ClC-2 Can Contribute to Cl− Secretion

Acknowledgments—We are grateful to Dr. T. Jentsch for the gift of rat ClC-2 cDNA and to Dr. E. Bugarrli for the gift of mouse ClC-4 cDNA. We also acknowledge the helpful discussions with Dr. Herman Yeger at the Hospital for Sick Children, Toronto.

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Studies showed that ClC-2 resides primarily in the membrane at this site (12). This distribution pattern is not typical for ion channels that have been implicated in chloride secretion. For example CFTR, the chloride channel thought to mediate chloride transport in many epithelial tissues, resides on the brush border membrane (24). Future studies are required to determine the molecular mechanisms that traffic ClC-2 to the apical aspect of the tight junction and those mechanisms that may act to retain the channel at this site. Finally, we have yet to determine whether there is a particular physiological significance for the concentration of an anion channel at the apical aspect of the tight junction. It is well known that the tight junction functions as a size and charge selective gate restricting the paracellular transit of organic and inorganic solutes (22, 23). Although our studies suggest that ClC-2, at the apical aspect of this junction, contributes to chloride secretion, its unique localization may also function to regulate the gate functions of the tight junction.

An understanding of the molecular basis for chloride secretion by epithelial tissues is key to identification of future therapies for intestinal secretory diseases such as diarrheal diseases and cystic fibrosis. Our studies indicate that ClC-2 contributes to the native secretory capacity of intestinal tissue, hence, modification of its function or location may affect the severity of secretory diseases.