Distribution of ClC-2 chloride channel in rat and human epithelial tissues

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The secretion of Cl− is critical for fluid and electrolyte transport across epithelia. The essential part of Cl− secretion is driven by the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) located in the apical membranes of secretory epithelia, such as those of the intestine and airways (1). Impaired or enhanced Cl− secretion results in diseases such as CF and secretory diarrhea (4, 5). The manifestations of CF result from a failure to secrete sufficient Cl−, so that mucosal surfaces are not adequately hydrated. There are, however, other, non-CFTR apical membrane Cl− channels that participate in epithelial Cl− secretion. In the pulmonary epithelium, Cl− secretion occurs through apical Ca2+-regulated Cl− channels (14). Although the presence of Ca2+-dependent Cl− channels in the apical membranes of enterocytes has not yet been confirmed, several findings point to the existence of a similar conductive pathway (4). Recent studies suggest that the ClC-2 Cl− channel, a member of the voltage-gated Cl− channel family, may be involved in Cl− secretion in rat fetal airway epithelial cells (7, 43) and in the small intestine from an unexpected location at the tight junction complex between epithelial cells (29, 41).

Northern blot analysis revealed that ClC-2 is widely expressed in mammalian tissues and in a variety of cultured cell lines (51). The expression of ClC-2 mRNA in Xenopus laevis oocytes induces currents that are slowly activated by strong hyperpolarization (51), a low extracellular pH (33), and hypotonicity (27). Similar currents are present in native cells, including epithelial cells and neurons (32). The biophysical properties and distribution pattern of ClC-2 have led to the suggestion that it helps regulate cell volume (23, 27, 58) and control intracellular Cl− concentration (46), in addition to its possible role in Cl− secretion. However, these suggestions must be confirmed, because a recent report indicated that ClC-2-deficient mice do not show spontaneous seizures or major changes in intestinal and lung anatomy and that the main abnormalities are severely degenerated retina and seminiferous tubules (8). The physiological role of ClC-2 in tissues other than the retina and testes may be taken by other proteins, and the question remains as to whether ClC-2 provides an alternative pathway for Cl− secretion in CF.

Epithelial ClC-2 distribution has so far been investigated only in rodents (29, 42, 45) and in a human intestinal cell line (41). The present study was therefore carried out to identify and locate the ClC-2 protein in human and rat tissues with a new polyclonal antibody (pAb-218) generated against a 16-amino acid sequence [amino acids (AA) 847–862] within the COOH-terminal region of ClC-2. The peptide sequence was
selected because of its amphipathy and possible antigenicity, because the amino acids in the rat and human peptides are identical, and because there are no conserved amino acids in other ClC proteins. A different anti-ClC-2 antibody (Clcn2), raised against the COOH terminus of ClC-2 (AA 888–906), became commercially available while our work was in progress. We therefore used both antibodies for comparative analysis of ClC-2 tissue distribution.

MATERIALS AND METHODS

Rat and Human Tissues

Lung and intestinal tissues were taken from 8-wk-old male rats (Sprague-Dawley) fed a standard diet. Samples of human lung were obtained at surgery from patients undergoing resection for lung carcinoma (Department of Pathology, Hôpital Laennec, Paris, France). Surgical specimens of normal human intestinal tissue were obtained from the Department of Pathology, Hôpital Necker-Enfants Malades (Paris, France). Only resected regions considered to be histologically normal were analyzed. Nasal samples were obtained from healthy adult volunteers. The human tissues were analyzed after obtaining informed consent and approval by the Institutional Ethics Committee of Hôpital Necker-Enfants Malades.

Cell Culture and Transient Expression of ClC-2 in HeLa Cells

T84 cells were cultured as previously described (22). Briefly, cells were grown at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM)-Ham’s F-12 medium (1:1 vol/vol; Life Technologies) containing 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 170 μg/ml streptomycin. For immunocytochemistry, cells were cultured for 48 h at low density on thin glass coverslips.

HeLa cells were grown in 35-mm culture dishes at 37°C with 5% CO₂ in DMEM (Glutamax; Life Technologies) containing 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Subconfluent cells were transfected with LipofectAMINE Plus reagent (Life Technologies) according to the manufacturer’s instructions. Rat ClC-2 cDNA (kindly provided by Prof. T. Jentsch, Hamburg University, Hamburg, Germany) was inserted into the expression plasmid pTracer (Invitrogen), a vector designed for the visual detection of transfected mammalian cells by green fluorescent protein (GFP). All experiments were performed 48 h after transfection.

Membrane Preparation

Polyclonal antibodies were generated in New Zealand White rabbits against a peptide corresponding to AA 847–862 (AIEGSVTAQGVKVRPP) of the COOH-terminal region of rat ClC-2 coupled to KLH (AgroBio). The peptide was selected after analysis of sequences as previously published (24, 31). Briefly, the sequence amphipathy was analyzed with the algorithm of Eisenberg, and a hydrophobic cluster analysis was used to identify a fragment with a high probability to lie at the protein-water interface. The selected fragment had the same sequence in the rat and human ClC-2 isoforms. The sequence of the peptide was screened to ensure that it was not homologous to that of any other member of the ClC family, minimizing the cross-reactivity of anti-ClC-2 antibody. No homology was found by using the protein data bank SWISSPROT. The serum obtained from the final bleed was affinity-purified on a peptide column prepared by covalently linking the peptide to N-hydroxysuccinimide-activated Sepharose (NHS-activated HiTrap, Amersham Pharmacia Biotech). The antibodies were eluted from the column, dialed, concentrated using Centricon 10 (Amicon), and frozen at −80°C. Polyclonal antibodies (pAb-218) had a high titer against the peptide on ELISA. The specificity and immunoreactivity of the purified antibodies were tested by immunoprecipitation and immunocytochemistry.

Membrane Preparation

Young male Sprague-Dawley rats (5–8 wk old) were killed by decapitation, and their brain, lung, small intestine, and colon were rapidly removed. All subsequent manipulations were carried out on ice. The intestinal epithelium was scraped off, and all the tissues were homogenized in a glass homogenizer in 10 volumes of sucrose buffer (250 mM sucrose, 20 mM HEPES, pH 7.4) containing protease inhibitors (protease inhibitor cocktail; Boehringer Mannheim). Tissue homogenates were centrifuged at 1,500 g for 10 min to remove cell debris. The supernatant was ultracentrifuged at 15,000 g for 40 min, and the resulting supernatant was ultracentrifuged at 100,000 g for 1 h. The final pellet was suspended in buffer containing (in mM) 10 KCl, 1.5 MgCl₂, and 10 Tris-HCl, pH 7.4. Protein concentration was determined by a modified Lowry method (40). The proteins (8–15 mg/ml) were then frozen and stored at −80°C.

Microsomes were prepared from transfected HeLa cells as described previously (18). Briefly, the cells were scraped off into ice-cold PBS, lysed in buffer containing (in mM) 10 KCl, 1.5 MgCl₂, and 10 mM Tris-HCl, pH 7.4, supplemented with protease inhibitor cocktail, and homogenized with a glass homogenizer. The homogenate was centrifuged at 200 g for 10 min to remove unbroken cells, and the resulting supernatant was ultracentrifuged at 100,000 g for 1 h. The final pellet containing the plasma membranes was suspended in lysis buffer, and the protein concentration was measured with the modified Lowry procedure. The proteins (5–10 mg/ml) were frozen and stored at −80°C.

Transfected HeLa cells were labeled with Redivue Pro-mix [35S] in vitro labeling mix, containing 35S-labeled L-methionine and 35S-labeled L-cysteine (150 μCi/ml for 3 h; Amersham Pharmacia Biotech). Cells were then lysed, and the proteins were dissolved in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail. Cell lysates were precooled by incubation with 10% Pansorbin suspension (Calbiochem) for 30 min. Staphylococcus aureus pellets were pelleted (12,000 g, 5 min), and the resulting supernatant (500 μl) was incubated with 1 μg of purified anti-ClC-2 antibody pAb-218 for 1 h. The samples were washed, denatured in SDS sample buffer, separated on 7.5% SDS-PAGE gel, and visualized with a PhosphoImager (Amersham Pharmacia Biotech). Controls were prepared by the same procedure except that the antibody incubation step was omitted.

35S Metabolic Labeling and Immunoprecipitation

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Immunoblotting

Rat membrane proteins (400 μg), protein extracts (200 μg) from transiently transfected HeLa cells, or protein extracts (600 μg) from HEK293 cells stably transfected with human (h)ClC-3 or hClC-5 were suspended in RIPA buffer and processed for immunoprecipitation as described in \( ^{35}S \text{ Metabolic Labeling and Immunoprecipitation } \). Precipitated proteins were separated on 7.5% SDS-PAGE and transferred to nitrocellulose filters (Bio-Rad). Free binding sites were blocked with 1% non-fat dry milk-1% bovine serum albumin (BSA) and 0.05% Tween 20 in Tris-buffered saline (TBS; in mM: 10 Tris-HCl, 150 NaCl, pH 8.0), and the membranes were probed with pAb-218 (diluted 1:5,000) or Clcn2 antibody purchased from Alomone Labs (diluted 1:250) and incubated overnight at 4°C. Nitrocellulose membranes were washed three times with TBS-T (0.05% Tween 20 in TBS) and incubated with goat anti-rabbit IgG(H+L) horseradish peroxidase (HRP)-conjugated (diluted 1:5,000; Biosys). Precipitated proteins were detected by incubating the nitrocellulose filters in ECL Plus reagent (Amersham Pharmacia Biotech) according to the manufacturer’s instructions and exposing them to Kodak X-ray film.

Immunohistochemical Procedures

Cells and cryosections of rat and human tissues were fixed in cold acetone for 10 min at 4°C and stored at –80°C. Two other fixatives, ethanol 95%-5% acetic acid and 4% paraformaldehyde, were also tested. Cells and sections were rehydrated in PBS, pH 7.4 and permeabilized with 0.1% Triton X-100 in PBS (PBSTM). Non specific binding sites were blocked with PBS-T, containing 3% BSA and 10% normal goat serum for 1 h at room temperature. Cells and sections were then incubated with primary antibodies overnight at 4°C (working dilutions: 1:1,000, 1.2 μg/ml for pAb-218 and 1:250, 10 μg/ml for Clcn2) and washed three times in PBS-T for 15 min each. They were then incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes), diluted 1:1,000 in 1% BSA in PBS-T, for 1 h at room temperature, washed several times, and mounted in Vectashield medium containing propidium iodide (Vector Laboratories). Double immunolabeling experiments were performed with the pAb-218 antibody in combination with monoclonal antibodies against rat macropheages (MAS 369; Harlan SeraLab, Loughborough, UK), E-cadherin (G-10; Santa Cruz Biotechnology), and zonula occludens-1 (ZO-1, clone ZO1–1A12; Zymed Laboratories). Anti-macrophage (diluted 1:250), anti-E-cadherin (diluted 1:50), and anti-ZO-1 (diluted 1:25) antibodies were visualized with Alexa 594-conjugated goat antimouse IgG(H+L) secondary antibody (Molecular Probes) diluted 1:1,000. Tissue sections were examined under a Zeiss confocal laser scanning microscope with argon ion lasers appropriate for FITC, Alexa 594 fluorochrome, and propidium iodide. Images were collected with Zeiss ×40 or ×100 oil objectives. Transfected HeLa cells were cultured on glass coverslips on which a grid was drawn with a diamond. This allowed easy relocation of individual cells, i.e., identification of transfected cells by GFP fluorescence and visualization of the same cells after fixation and after staining with ClC-2 antibodies. Fluorescence images of transfected HeLa cells were photographed through a ×50 oil plan Achromat objective using a Nikon camera attached to a TE 300 Nikon microscope equipped with an optimized GFP filter set. Transfected HeLa cells stained with ClC-2 antibodies were also examined by confocal laser scanning microscopy, and images in the x-y or x-z (collected at 1.8-μm intervals) planes were stored on hard disk.

Negative controls were routinely performed in parallel by omitting the primary antibody or by peptide competition. For the latter, ClC-2 antibodies in the working dilutions used were preincubated for 1 h at room temperature with a fivefold excess of the corresponding peptide antigens. Specific staining and negative controls were photographed under identical conditions. All final images were prepared with Paint Shop Pro and Adobe Illustrator software.

RESULTS

Generation and Characterization of Anti-CLC-2 Antibodies

The specificity of the two antibodies, pAb-218 and Clcn2, was determined with immunoprecipitation and immunocytochemistry. Western blot analysis of extracts from HeLa cells transiently transfected with ClC-2 cDNA revealed that pAb-218 antibody did not detect ClC-2 protein, presumably because of a weak interaction with the SDS-denatured protein. Thus the specificity of anti-CLC-2 antibody was first assessed by immunoprecipitation of metabolically labeled HeLa cells (Fig. 1A). A 90- to 97-kDa protein band, corresponding to the predicted molecular mass of ClC-2, was detected (Fig. 1A, lane 4), whereas no band was seen in mock-transfected cells (lane 3); nonspecific IgGs puriﬁed from preimmune serum did not detect this band (lane 2). The background was due to nonspecific binding of \( ^{35}S \)-methionine/cysteine-labeled proteins to the protein A beads, as conﬁrmed by omitting the antibody (Fig. 1A, lane 1). pAb-218-immunoprecipitated proteins could also be detected with anti-CLC-2 antibodies. As shown in Fig. 1B, the pAb-218-immunoprecipitated ClC-2 protein was recognized by both pAb-218 (lane 2) and Clcn2 (lane 3).

The suitability of both antibodies for immunocytochemistry was tested in HeLa cells transiently transfected with ClC-2 cDNA (Fig. 1C). Visualization of GFP fluorescence in living cells before immunoprocessing conﬁrmed that only transfected cells were labeled by the anti ClC-2 antibodies. As described in MATERIALS AND METHODS, GFP-positive cells were identiﬁed and photographed before immunoprocessing (Fig. 1C, a and d). The same cells were photographed after staining with pAb-218 (Fig. 1C, c) or Clcn2 (Fig. 1C, e). The GFP fluorescence almost completely disappeared during cell ﬁxation, as illustrated in Fig. 1C, b, and thus could not overlap with the green ﬂuorescence of the secondary antibody. Confocal images obtained in x-y and x-z planes (Fig. 1C, f–i) showed that ClC-2 labeling was not restricted to the plasma membrane, which can be expected with an overexpressing system.

Although the ClC-2 antibodies used in the present study were raised against epitopes on the basis of no similarity to other ClC isoforms, we tested a possible cross-reactivity of the ClC-2 antibodies with two other isoforms, ClC-3, which is broadly expressed (32), and ClC-5, which, besides its predominant expression in kidney cells, has been demonstrated to be present in rat intestinal cells (52).

In immunoprecipitation experiments, a slight cross-reactivity to ClC-3 and ClC-5 was detected, but only
when high concentrations of total protein were used (concentrations were 3-fold higher than those used for ClC-2 detection). Cln2 antibody immunoprecipitated proteins of ~85 (Fig. 1D, left) and 100 (Fig. 1E, left) kDa, consistent with the predicted mass of hClC-3 and of hClC-5 tagged with GFP protein, respectively. pAb-218 antibody did not immunoprecipitate ClC-3 protein (Fig. 1D, lane 2) but detected the ClC-3 immunoprecipitated by Cln2 antibody (Fig. 1D, lane 1). A slight cross-reactivity to ClC-5 protein was observed with pAb-218 (Fig. 1E, lane 2).

On the other hand, immunostaining of HEK293 cells stably expressing ClC-3 or ClC-5 was negative with pAb-218 (Fig. 1, D, a and E, a, for ClC-3 and ClC-5, respectively) and with Cln2 (Fig. 1, D, b and E, b, for ClC-3 and ClC-5, respectively).

Expression of ClC-2 in Rat Tissues

The ClC-2 in rat tissues was analyzed by immunoprecipitation followed by immunoblotting with pAb-218, because tissue samples cannot be metabolically labeled. We first assessed the specificity of pAb-218 in rat brain membranes because CIC-2 is known to be abundant in this tissue (15). As expected, pAb-218 recognized a single 90- to 97-kDa protein band (Fig. 1F, lane 1). A protein of ~97 kDa was also immunoprecipitated from membrane preparations of rat colon, small intestine, and lung and detected with pAb-218 (Fig. 1F, lanes 2–4) or with Cln2 antibody (not shown).

Immunohistochemical Localization of ClC-2 in Rat and Human Tissues

All the tissues shown were fixed in acetone because this gave optimal fluorescent signals. Tissues fixed in paraformaldehyde were unsuitable for cytoimmunochemistry of the protein, perhaps because the epitope involves lysine and arginine, two amino residues that are modified by these fixatives. Ethanol-fixed tissues showed no labeling.

**Rat colon and small intestine.** Both antibodies predominantly stained luminal enterocytes in the rat colon (Fig. 2A, a and c), whereas preabsorption of the antibodies with their corresponding peptide antigens resulted in no staining (Fig. 2A, b and not shown). The staining with pAb-218 appeared to be mainly on the basolateral membranes and to a lesser extent in the cytoplasm (Fig. 2A, d). Similar results were obtained with Cln2, but with lower resolution of the basolateral membranes and more diffuse cytoplasmic labeling (Fig. 2A, g). ClC-2 staining was weaker in the cells lining the crypts and was observed only in the upper two-thirds of the crypts. The basolateral membranes were faintly labeled by the pAb-218 antibody (Fig. 2A, e), but little or no staining was detected with Cln2 (Fig. 2A, f).

Both antibodies stained the enterocytes along the villi of the rat small intestine (Fig. 2B, a and d), but there was no labeling in the crypts (not shown). The lateral membranes limiting the intercellular spaces were distinctly stained with pAb-218, and a weak diffuse labeling in the cytoplasm was also observed (Fig. 2B, b). A control section (Fig. 2B, c) demonstrates that preincubation with pAb-218 peptide blocks immunolabeling. The Cln2 antibody gave comparable but less intense staining on the lateral membranes associated with patchy cytoplasmic or apical labeling in some cells (Fig. 2B, d).

**Human colon and small intestine.** There was significant ClC-2 immunostaining in the enterocytes lining the luminal surface of the human colon, but the subcellular staining pattern differed from that observed in the rat. The expression of ClC-2 was not present at the basolateral membranes, but pAb-218 mainly stained a cytosolic supranuclear region (Fig. 3a), and a discrete, punctate staining was detected at the apical pole (Fig.
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A

B

IP preimmune serum + - -
IP pAb-218 - + -
Blot pAb-218 - + -
Blot Clcn2 - - +

C

D

E

F

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C809

CLC-2 IN INTESTINAL AND LUNG EPITHELIUM

A

B

IP preimmune serum + - -
IP pAb-218 - + -
Blot pAb-218 - + -
Blot Clcn2 - - +

C

D

E

F

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Omission of the primary antibody or application of preabsorbed antibody yielded no detectable signal (Fig. 3b). Staining with Clcn2 was similar but less intense (Fig. 3c). Intracellular ClC-2 was also detected with pAb-218 in the upper two-thirds of the crypts (Fig. 3d), but cells at the base of the crypts were not stained (Fig. 3e). The punctate apical staining pattern observed with both antibodies was reminiscent of ClC-2 staining at the tight junction complex, previously described in the murine small intestine (29). However, we did not observe colocalization of ClC-2 with the tight junction protein ZO-1, indicating a different subcellular expression at or near the apical membrane (Fig. 3f). Figure 3, g and h, shows labeling of the lateral membranes by an antibody against the adhesion protein E-cadherin. Hence, the absence of ClC-2 expression at the basolateral membranes as observed in rat colonic cells could not simply be explained by degradation of the tissue.

The subcellular distribution of ClC-2 was also examined in T84 human colon cells, in which ClC-2 currents have been described (22). Immunoreactive ClC-2 was detected only in a fraction of isolated cells, and the labeling pattern varied from cell to cell. ClC-2 was seen as a rim around the periphery of some cells, presumably at or beneath the plasma membrane (Fig. 3i), whereas the signal was mostly intracellular in others (Fig. 3j).

ClC-2 protein localization in the human small intestine was difficult because of background staining that remained after incubation of the sections with preabsorbed pAb-218. Moreover, a nonspecific and strong labeling of the nuclei was obtained with the Clcn2 antibody (not shown). For these reasons, no conclu-
sions can be drawn about CIC-2 expression in this tissue.

**Rat and human airway epithelium.** CIC-2 staining with the pAb-218 antibody was mainly localized in the apex of the ciliated cells in the rat bronchial epithelium (Fig. 4A, a and b). The punctate labeling of the peribranchial connective tissue was not specific, because it was also present in control sections (Fig. 4A, c). The Clcn2 antibody stained the same structures of the ciliated cells, but the signal was much weaker (Fig. 4A, d).

The apical surface of bronchiolar epithelial cells was also stained with pAb-218 (Fig. 4A, e and g). The labeled cells were difficult to identify because of poor preservation of the tissue morphology in cryosections, but the CIC-2 antibody seemed to bind primarily to the...
ciliated cells (Fig. 4A, g and h). There seemed to be more nonspecific staining of the peribronchiolar connective tissue with Clcn2, whereas the epithelial staining was very weak (Fig. 4A, i).

A subset of alveolar cells, probably corresponding to type II pneumocytes or macrophages, was specifically labeled with anti-ClC-2 antibodies (Fig. 4A, j–m). Double immunofluorescence labeling with pAb-218 and a
monoclonal anti-rat macrophage antibody (MAS 369) revealed that the ClC-2 (green) and the MAS 369 (red) signals were never observed in the same cells. These results suggest that the ClC-2-expressing cells represent type II pneumocytes (Fig. 4A, l).

The pAb-218 antibody also gave significant apical staining of the ciliated cells of human bronchi and bronchioles (Fig. 4B, a and not shown, respectively), whereas preabsorbed antibody gave no fluorescent signal (Fig. 4B, b). A high-power view (Fig. 4B, c) of the ciliated cells showed that ClC-2 was not restricted to the apical membrane but was also present in submembrane compartments. The Clcn2 antibody bound far less to these structures, as in the rat (Fig. 4B, d). ClC-2 was also detected in the cytoplasm and at the apex of ciliated cells of human nasal samples (Fig. 4B, f). Some of the human alveolar cells were also well labeled with pAb-218 (Fig. 4B, h) and weakly stained with Clcn2 (Fig. 4B, i). These cells have not yet been identified, but they may represent type II pneumocytes, as in the rat. The bronchial submucosal glands were not labeled with either antibody (not shown).

**DISCUSSION**

In this work we have analyzed the pattern of ClC-2 distribution in intestinal and airway epithelia. The main tool used was a new polyclonal antibody, pAb-218, raised against a COOH-terminal sequence (AA 847–862) that is identical in the rat and human ClC-2. Of note, the rat sequence (AA 888–906) used to raise the other antibody tested, Clcn2, differs by two amino acids from the human CIC-2. Clcn2 detected the denatured protein (11), whereas pAb-218 antibody preferentially recognized the native conformation of CIC-2. Neither pAb-218 nor Clcn2 recognized ClC-3 and ClC-5 isoforms in immunocytochemistry experiments. In immunoprecipitation experiments, a slight cross-reactivity with the two ClC isoforms was observed for both anti-CIC-2 antibodies but only when immunoprecipitation was performed with high amounts of ClC-3 or ClC-5.

There were few differences in the subcellular localization of ClC-2 labeling with the two antibodies; however, within the same cellular structures, staining with pAb-218 was more accentuated.

**Intestine Epithelia**

In the rat, both antibodies stained the villus cells of the small intestine and the surface enterocytes of the colon. It has been reported that the expression of ClC-2 in the rat intestine is downregulated during late gestation (42), but Gyömörey et al. (29) detected ClC-2 in the small intestine of 6- to 8-wk-old mice. Although we did not compare fetal and adult tissues, our results agree with this latter study and further demonstrate that ClC-2 protein is significantly expressed in intestinal tissues of adult rats. However, the subcellular distribution of ClC-2 reported here differs from that reported for the mouse (29). Those authors used an antibody generated against a NH2-terminal epitope and detected ClC-2 predominantly at the tight junction complex between the enterocytes of the small intestine (29). The discrepancy between this restricted distribution and the expression all along the lateral membranes found by us may be explained by different patterns of ClC-2 gene expression in the rat and mouse or by differences in the sensitivities of the antibodies used. We detected an intense fluorescent signal with pAb-218 at an antibody concentration 50-fold lower than that used in the mouse sections, but, in agreement with the preliminary observations of Gyömörey et al., we found a high level of ClC-2 expression at the basolateral membranes of the rat colon luminal cells. The labeling by both antibodies was found at the lateral or basolateral membranes; the labeling by Clcn2, but not by pAb-218, also extended to apical material in the villus cells or intracellular material in the luminal colon cells. Because pAb-218 did not stain these subcellular compartments, it remains difficult to conclude whether Clcn2 was more sensitive for detection of ClC-2 in the rat intestine or whether it additionally bound to cross-reactive material. Because Clcn2 antibody did not stain ClC-3 and ClC-5-expressing HEK cells, it is unlikely that the cross-reactive material corresponds to these isoforms, which can be expressed in the gut (32, 52).

Our results reveal important differences between the subcellular distribution of ClC-2 in the rat and human intestine. The protein was found in surface colon enterocytes in both species, but ClC-2 immunolabeling was mainly present in an apically oriented perinuclear compartment, associated with discrete and punctate ClC-2 labeling at the apex of human colon cells. This pattern may indicate accumulation of the protein in the Golgi apparatus or in a distinct compartment involved in the polarity-dependent targeting of proteins (53). This is in keeping with our recent suggestion (3) that the number of ClC-2 channels in the

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**Fig. 4.** ClC-2 in the rat (A) and human (B) airways. A: sections of rat bronchi incubated with pAb-218 (a and b), preabsorbed pAb-218 (c), and Clcn2 (d). h: High-magnification view of the section shown in a. ClC-2 immunolabeling is detected at the apex of the ciliated cells and at the top of the cilia. e–i: sections of rat bronchioles incubated with pAb-218 (e and g), preabsorbed pAb-218 (f), and Clcn2 (i). The apical membrane staining shown in e seems to be mainly in ciliated cells (arrowheads) as shown at a higher magnification (g and h). Clcn2 gives little apical staining but a strong positive signal in the peribronchiolar connective tissue (i). j–m: ClC-2 in alveoli. A subset of cells is labeled with pAb-218 (j) and Clcn2 (m). Double-staining experiments (l) show that anti-macrophage antibody (red signal) does not bind to ClC-2-expressing cells (green signal). B: sections of human bronchi incubated with pAb-218 (a), preabsorbed pAb-218 (b), Clcn2 (d), and preabsorbed Clcn2 (e). pAb-218 gives strong labeling of ClC-2 at the apex of the cells. c: A higher magnification shows a fine punctate submembranar labeling. f: ClC-2 staining of ciliated cells from human nasal samples. g–i: ClC-2 in gobulose cells of alveoli: pAb-218 (h), phase-contrast view of the section shown in g (g), and Clcn2 (i). Bars, 20 μm except for A, b and B, c (5 μm).
membrane of human intestinal T84 cells may be regulated by vesicular transport. In addition, the intracellular localization of CIC-2 may point to a role in pH regulation in intracellular compartments, as demonstrated for other members of the CIC gene family (28, 36, 48).

The reason for the different distributions of CIC-2 expression in rat and human luminal colon cells is unknown. The overall amino acid sequences of the rat and human isoforms are 93% identical but differ in a putative protein kinase (PKA) consensus site in the COOH-terminal region of the human isoform, which is absent from the rat CIC-2 protein (13, 51). There are several examples of phosphorylation by PKA acting as a mechanism for protein targeting (2, 34, 54). Perhaps this phosphorylation site contributes to the different localization of the CIC-2 protein in human intestinal tissues, which would suggest different functions.

The lack of CIC-2 staining at the membranes of human crypt colonocytes apparently contradicts functional data demonstrating CIC-2 currents in two human intestinal cell lines, T84 cells (22), which are representative of secretory crypt cells (19), and Caco-2 cells (41), which have been used to model absorptive as well as secretory functions (6, 25). These results may suggest that the density of CIC-2 channels is lower in native and normal crypt cells. However, it should be noted that the amplitude of the CIC-2 current in T84 cells varies from one cell to another, being smaller than −100 pA at −120 mV in most cells (22). The variable pattern of CIC-2 labeling in T84 cells demonstrated here is thus compatible with the various current amplitudes reported previously and with the absence or very low expression of CIC-2 in native crypt cell membranes.

In the rat, the high level of basolateral expression of CIC-2 channels in absorptive cells of the villi and colon is more consistent with a role for these channels in NaCl absorption than secretion. Electroneutral NaCl absorption is the primary absorption process in surface cells of the rat colon and enterocytes of the villi, via the coupling of Na+/H+ exchanger (NHE)2/3 and Cl−/anion exchange. The luminal uptake of substrates for Na+ coupled transport during absorption causes the cell to swell, leading to volume regulatory activation of K+ and Cl− channels at the basolateral membrane. The activation of these channels not only limits cell swelling but maintains the electrical driving force for continuous transport (44). Thus, in accord with their osmotic sensitivity, CIC-2 channels at the basolateral membrane may contribute to cell volume regulation and Cl− exit during NaCl absorption. It is also interesting that intestinal epithelial cells maintain pH microclimates both outside and inside their plasma membranes and that the lateral intercellular spaces and external basal domains are slightly acidic (pH ~6.7), whereas their intercellular counterparts are more alkaline (12, 39). These pH gradients may be necessary for activation of NHEs and Na+ absorption (39). Whether CIC-2 plays a role in regulating the pH and ionic composition of these microenvironments remains to be determined, but its location in basolateral membranes fits well with its activation by extracellular acidification or intracellular alkalization.

In contrast to the rat, the presence of CIC-2 in the apex of human luminal colonocytes, although relatively low, suggests that the channel may contribute to Cl− backleak in the secretory direction, hence opposing the net transepithelial Cl− absorption, which is predominant at this site.

Airways

CIC-2 is present in ciliated cells and probably in alveolar type II pneumocytes of rat and human adult airways. These findings disagree with previous reports showing a marked downregulation of CIC-2 mRNA and protein in the rat lung after birth (43). A recent study also showed that the amount of CIC-2 mRNA in the human pulmonary epithelium declines by the end of pregnancy and remains relatively low at all postnatal times (37). As discussed above, detection of the protein in adult tissues may be due to the great sensitivity of the pAb-218 antibody.

The distribution of CIC-2 along the luminal membrane of developing airways may be taken as evidence that CIC-2 contributes to Cl− secretion (43), which is the dominant ion transport in the fetal lung (16, 59). It has been also proposed that the relative acidic pH of fetal lung fluid may lead to its activation (7). However, a CIC-like hyperpolarization-activated Cl− current, Ihyp-act, whose sensitivity to extracellular pH differs from that of CIC-2, has been identified in adult murine nasal ciliated cells (50). Ihyp-act is fully active over the pH range 5.4–9 and inhibited only when extracellular pH is reduced to 5.0. This sensitivity resembles that of members of the second branch of the CIC Cl− channel family (21). The current-voltage relationship of Ihyp-act also differs from those of CIC-2 and other endogenous or expressed CIC channels. Because CIC-2, CIC-3, and CIC-6 mRNAs have been described in tracheal epithelia (37), Ihyp-act and the protein detected in the ciliated cells may represent a functional heterooligomeric channel with different properties (38).

We found that CIC-2 expression in the ciliated cells was not restricted to the apical membrane, which is further evidence that the number of channels in the plasma membrane may be regulated by vesicle trafficking. It is well established that the airway surface epithelium essentially absorbs NaCl under normal conditions after birth. Although the mechanisms of NaCl absorption by airway and intestine epithelia differ, it remains intriguing that CIC-2 channels are mainly present in those cells involved in fluid absorption. This raises the question of their role in the regulation of this process. The presence of the anionic exchanger DRA in the trachea (56) and the need for an apical mechanism for removing HCO3− from alveolar cells (17) suggest that CIC-2 is important for regulating the environmental pH of the epithelial airway cells, as for the intestine, testis, and retina.
CIC-2 as Alternative Chloride Pathway in CF

Our findings that there is no CIC-2 in human submucosal glands or in secretory enterocytes of the deep crypts, which are the major sites of CFTR expression (20, 49) may call into question the relevance of CIC-2 activation in bypassing defective CFTR to ensure sufficient Cl− and fluid secretion. However, Cl− can be secreted across the surface airway epithelium through CFTR or alternate Cl− conductance pathways under some experimental conditions in vitro, when Na+ entry is blocked (9, 57). This probably allows regulation of the salt and water contents of the periciliary liquid. Of interest, the pattern of CIC-2 distribution seems to overlap that of CFTR in ciliated cells (10). Some experimental data also suggest that Cl− secretion may not be confined to the crypts in the intestine but can take place via surface epithelia (35, 47). Thus the presence of CIC-2 at the apical region of ciliated cells, and to a lesser extent at the apex of human enterocytes, supports the idea that activation of CIC-2 may provide an alternate pathway for Cl− secretion when CFTR is absent. Our findings also indicate that special attention must be taken when choosing the mammalian species to investigate CF therapies involving CIC-2 activation. The basolateral location of CIC-2 in rodent intestinal tissues (this report and Ref. 29) and the absence of major pulmonary pathophysiology in the CF mouse (26) suggest that mouse may not be an appropriate experimental model.

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REFERENCES


28. Günther W, Lüchow A, Cluzeaud F, Wandevalle A, and Jentsch TJ. CIC-5, the chloride channel mutated in Dent's

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