Tissue distribution and subcellular localization of the ClC-5 chloride channel in rat intestinal cells

ALAIN VANDEWALLE,1 FRANÇOISE CLUZEAUD,1 KOU-CHENG PENG,1 MARCELLE BENS,1 ANKE LÜCHOW,2 WILLY GÜNThER,2 AND THOMAS J. JENTSCH2

1Institut National de la Sante et de la Recherche Médicale, Unité 478, Institut Fédératif de Recherche 02, Faculté de Médecine Xavier Bichat, BP 416, 75870 Paris Cedex 18, France (E-mail: vandewal@bichat.inserm.fr)
2Zentrum für Molekulare Neurobiologie Hamburg, Universität Hamburg, Martinistrasse 85, D-20246 Hamburg, Germany

Received 9 May 2000; accepted in final form 6 September 2000

Vandewalle, Alain, Françoise Cluzeaud, Kou-Cheng Peng, Marcelle Bens, Anke Lüchow, Willy Günther, and Thomas J. Jentsch. Tissue distribution and subcellular localization of the ClC-5 chloride channel in rat intestinal cells. Am J Physiol Cell Physiol 280: C373–C381, 2001.—ClC-5 is the Cl– channel that is mutated in Dent’s disease, an X-chromosome-linked disease characterized by low molecular weight proteinuria, hypercalciuria, and kidney stones. It is predominantly expressed in endocytically active renal proximal cells. We investigated whether this Cl– channel could also be expressed in intestinal tissues that have endocytotic machinery. ClC-5 mRNA was detected in the rat duodenum, jejunum, ileum, and colon. Western blot analyses revealed the presence of the 83-kDa ClC-5 protein in these tissues. Indirect immunofluorescence studies showed that ClC-5 was mainly concentrated in the cytoplasm above the nuclei of enterocytes and colon cells. ClC-5 partially colocalized with the transcytosed polymeric immunoglobulin receptor but was not detectable together with the brush-border-anchored sucrase isomaltase. A subtraction of vesicles obtained by differential centrifugation showed that ClC-5 is associated with the vacuolar 70-kDa H+-ATPase and the small GTPases rab4 and rab5a, two markers of early endosomes. Thus these results indicate that ClC-5 is present in the small intestine and colon of rats and suggest that it plays a role in the endocytotic pathways of intestinal cells.

The ClC-5 Cl– channel, encoded by the CLCN5 gene, belongs to the family of voltage-gated Cl– channels. This gene family is composed so far of nine members in mammals (see Ref. 19 for review). The first ClC-0 channel was identified by Jentsch et al. (20) by expression cloning from the marine ray Torpedo marmorata. ClC-1 is the major Cl– channel in skeletal muscle, which is mutated in both the dominant and recessive forms of myotonia (38). ClC-2 is present in many tissues and is thought to play a role in cell volume regulation (14). The rat rClC-K1 and rClC-K2 (hClC-Ka and hClC-Kb in humans) channels are specific to the kidney (23, 41). Mutations of the hCLCN-KB gene lead to Bartter’s syndrome type III (35), whereas mice in which the CIC-K1 gene has been knocked out develop nephrogenic diabetes insipidus (26). The CIC-3 and CIC-4 channels are broadly expressed and are very similar (almost 80% identical) to ClC-5 (39). In contrast, the ubiquitous putative CIC-6 and CIC-7 Cl– channels are only ~30% identical to the other ClC channels (19).

Mutations in the human CLCN5 gene have been found to occur in Dent’s disease, an X-linked hereditary hypercalciuric nephrolithiasis, causing low-molecular-weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, progressive renal insufficiency, and in some cases rickets (see Ref. 33 for review). This discovery has stimulated research to obtain a better understanding of the function of ClC-5 in the kidney. Cloning and functional expression studies in Xenopus oocytes have demonstrated that the rat ClC-5 channel elicits Cl– currents (39). Günther et al. (15) demonstrated that the 83-kDa rat ClC-5 Cl– channel is predominantly located in endocytotic vesicles underlying the apical membrane domain of kidney proximal and intercalated cells of the collecting duct. Others have found a similar distribution of ClC-5 in proximal tubule cells and intercalated cells as well as in thick ascending limb cells in the kidneys of rats, mice, and humans (9, 24, 32).

The colocalization of rClC-5 with vacuolar H+-ATPases and endocytosed β2-microglobulin (15) strongly suggests that the ClC-5 Cl– channel is essential for proximal tubule endocytosis by providing an electrical shunt for the acidification of endocytotic vesicles. These results also provide a molecular basis for the proteinuria observed in Dent’s disease. More recently, Luyckx et al. (25) have reported that transgenic mice with reduced ClC-5 expression due to the introduction of a CIC-5-inactivating antisense ribozyme transgene were slightly hypercalciuric when fed a normal Ca2+ diet. These authors also speculated that ClC-5 is expressed in the intestine and suggested that the hyper-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpcell.org 0363-6143/01 $5.00 Copyright © 2001 the American Physiological Society C373
calcium that occurs in ribozyme transgenic mice could be due to the hyperabsorption of Ca$^{2+}$ in the intestine. These results raised the question about the cellular and subcellular distribution and function of ClC-5 in the intestine. The differentiated epithelial cell lines lining the crypt-villus axis of the small intestine and colon have many morphological and functional features that are similar to the renal proximal tubule cells. They possess a well-developed apical brush border rich in membrane transporters and membrane-anchored hydrolases (22). A large number of in vitro studies have also demonstrated that epithelial polarized intestinal and kidney tubule cells have similar endocytotic systems for the uptake of peptides and the turnover of proteins residing in the apical membrane (4, 16, 17).

The present study was designed to analyze the expression of the ClC-5 gene and the cellular and subcellular distribution of this Cl$^-$ channel in rat intestinal and colon epithelial cells. RT-PCR and immunohistochemical studies showed ClC-5 mRNA and protein in the small intestine and colon cells. This Cl$^-$ channel was located in a vesicle-rich region beneath the apical brush border of enterocytes. The coexpression of ClC-5 with the 70-kDa subunit of the vacuolar-type H$^+\cdot$ATPase (29) and small GTPase rabs and with internalized polymeric immunoglobulin receptors (p IgRs) responsible for the transepithelial transport of dimeric immunoglobulin A (d IgA; see Refs. 2 and 36) also suggests that this Cl$^-$ channel plays a role in the endocytotic pathway(s) of epithelial intestinal cells.

**MATERIALS AND METHODS**

*Rat tissues.* Experiments were performed on gastrointestinal and kidney tissues from adult male rats fed a standard diet with free access to tap water. All tissue samples were rapidly frozen in liquid nitrogen and kept at $-80^\circ$C until used.

*RNA extraction and RT-PCR.* Total RNAs were extracted from rat duodenum, jejunum, ileum, proximal and distal colon, kidney, and skeletal muscle using the RNA-PLUS extraction kit (Quantum Biotechnologies, Illkirch, France). RNA (2 $\mu$g) was reverse-transcribed with Moloney murine leukemia virus RT at 42°C for 45 min. cDNA (100 ng) and non-reverse-transcribed RNA were amplified for 28 cycles in 100 $\mu$L total volume of PCR buffer (50 mM KCl and 20 mM Tris-HCl, pH 8.4) containing 40 $\mu$M dNTP, 2 mM MgCl$_2$, 1 $\mu$Ci [alpha-32P]dCTP, 1 unit *Taq* polymerase, 36 pmol of rat ClC-5 primers, and 11.5 pmol of human glyceraldehyde-3-phosphate dehydrogenase (h GAPDH; internal standard) primers. The rat ClC-5 primers used were those described by Steinmeyer et al. (39). The hGAPDH primers were those described by Hummeler et al. (18). The thermal cycling program was 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Amplified products were run on a 4% polyacrylamide gel and autoradiographed.

*Protein extraction and immunoblot analysis.* ClC-5 was detected by Western blotting using a rabbit polyclonal anti-CIC-5 antiserum, PEP5A2, previously characterized by Günther et al. (15). Briefly, PEP5A2 was raised in rabbits against a synthetic peptide (KSRDHRHREITNKS) representing a part of the amino terminus of ClC-5. This peptide was coupled to BSA by 3-maleimidobenzoic acid N-hydroxysuccinimide ester via a cysteine added to the amino terminus of the peptide and injected several times (in intervals of 3 wk) in rabbits. The serum obtained from the final bleed was purified by affinity chromatography against the peptide. The specificity was checked in Western blots using membranes from Xenopus oocytes previously injected with ClC-3, ClC-4, or ClC-5 and additionally in indirect immunofluorescence experiments using COS-7 cells transiently transfected with either one of these related channel cDNAs (15). The abdominal cavity of the killed rats was opened. Kidneys and lungs were removed and pulverized in liquid nitrogen. The intestine and colon were cannulated and rinsed with ice-cold PBS. Five- to seven-cm-long pieces of duodenum, jejunum, ileum, and colon were removed and placed in ice-cold PBS. The lumen was opened with a scalpel, and cells were gently scraped off, collected, centrifuged (150 g for 5 min at 4°C), and kept in liquid nitrogen until used. Tissue samples and pelleted cells were homogenized in a hypertonic sucrose solution (0.25 M sucrose, 3 mM imidazole, and 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 $\mu$g/ml protease inhibitor cocktail (Boehringer Mannheim) in a glass Dounce homogenizer (10 strokes at 4°C). All samples were kept at 4°C for 30–45 min and centrifuged at 150 g for 5 min at 4°C to remove nuclei and any remaining intact cells. The supernatant was then centrifuged (105,000 g for 1 h at 4°C). Pelleted enriched membrane preparations were then suspended in 250 $\mu$L sucrose buffer and were used for Western blotting.

Western blotting was also performed on subcellular fractions from rat jejunum prepared by differential centrifugation following the procedures described by Devuyst et al. (9) for the isolation of subcellular fractions from kidney. The jejunal was perfused with ice-cold PBS, and scraped-off cells were homogenized in a glass Dounce homogenizer (40 strokes at 4°C) in ice-cold homogenization buffer (300 mM sucrose and 25 mM HEPES, adjusted to pH 7.0 with 1 M Tris) containing 1 mM PMSF and 100 $\mu$g/ml protease inhibitor cocktail. All subsequent steps were performed at 4°C. The cell homogenate was centrifuged at 500 g for 20 min to remove debris. The resulting supernatant (S1) was centrifuged at 8,000 g for 30 min. The pellet, corresponding to the enriched membrane fraction, was suspended in the ice-cold homogenization buffer and centrifuged at 19,000 g for 20 min. Two steps of centrifugation were then performed on the pellet (P1) and supernatant (S2). The P1 pellet was suspended in homogenization buffer, layered on top of a 1.12 M sucrose and 25 mM HEPES, adjusted to pH 7.0 with 1 M Tris) containing 1 mM PMSF and 100 $\mu$g/ml protease inhibitor cocktail. The interface layer was centrifuged at 40,000 g for 20 min, and the resulting pelleted fraction (fraction I) containing plasma membranes was kept. The S2 supernatant was centrifuged at 42,000 g for 20 min. The resulting low-speed pellet (fraction II) was kept at $-80^\circ$C while the supernatant was centrifuged again at 100,000 g for 60 min. The interface layer was centrifuged at 40,000 g for 20 min, and the resulting pelleted fraction (fraction I) containing plasma membranes was kept. The S2 supernatant was centrifuged at 40,000 g for 20 min. The resulting low-speed pellet (fraction II) was kept at $-80^\circ$C while the supernatant was centrifuged again at 160,000 g for 75 min, and the resulting high-speed pellet (fraction III) was kept. The protein contents of the membrane-enriched tissue fractions and subcellular fractions I, II, and III were determined by the Bradford (5) method using BSA as standard. All extracts were kept at $-80^\circ$C until used.

Western blotting was performed using PEP5A2 alone or with an antibody against monoclonal antibody (Mab A-5441; Sigma, ST. Quentin Fallavier, France), a polyclonal antibody raised against the 70-kDa subunit (subunit B) of the bovine vacuolar H$^+\cdot$ATPase pump (kindly provided by Dr. D. K. Stone, Dallas, TX), a polyclonal antibody directed against E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), and polyclonal antibodies raised against rab4, rab5a, or rab6 (Santa Cruz Biotechnology). Membrane-enriched tissue fractions and subcellular fractions I, II, and III were subfraction-
ated by SDS-PAGE using a 7.5% (for PEP5A2 and the anti-E-cadherin and anti-H-ATPase antibodies) or 15% (for the anti-rab4, -rab5a, and -rab6 antibodies) resolving gels with 4% stacking gel. Proteins were transferred to polyvinylidene difluoride membranes in 25 mM Tris·HCl, 192 mM glycine, and 25% methanol. Rainbow molecular weight markers were used as size standards (Amersham). The membrane was blocked by incubation with 5% skim milk in TBS-T (10 mM Tris·HCl, pH 8.5, 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature. The membrane was probed with PEP5A2 (1:2,000), the anti-β-actin Mab (1:10,000), the anti-H-ATPase antibody (1:5,000), the anti-E-cadherin antibody (1:200), or the anti-rab antibodies (1:2,000) by incubation for 2 h at room temperature. It was then incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG; Dako, Trappes, France) for 1 h at room temperature. Negative controls were performed by omitting the primary antibodies. The membrane was washed with TBS-T, and the antigen-antibody complexes were detected with the enhanced chemiluminescence Plus Western blotting detection system (Amersham Pharmacia Biotech Europe, Orsay, France).

**Immunofluorescence studies.** Rats were anesthetized by an intraperitoneal injection of nembutal. Fragments (1 cm long) of duodenum, jejunum, ileum, and a midportion of the colon were rinsed in PBS, snap-frozen in liquid nitrogen, and stored at −80°C until used. Kidneys were removed rapidly, cut along the longitudinal axis of the medullary rays, and processed as described above. Frozen tissues were sectioned (5–7 μm thick) with a cryostat (Bright). Tissue sections were mounted on superfrost glass slides and fixed in ice-cold methanol for 8 min. Samples were incubated with the anti-PEP5A2 CIC-5 antibody for 2 h at room temperature, rinsed three times with PBS, and incubated with goat anti-rabbit IgG coupled to Cy3 reactive dye (Cy3; Jackson Immunoresearch) for 1 h at room temperature. The sections were rinsed in PBS and mounted. The specificity of the labeling was checked by indirect immunofluorescence on jejunal sections by diluting the primary PEP5A2 antibody with an excess (1.15–11.5 μg/ml) of the synthetic peptide used to produce PEP5A2. Negative controls were also performed by omitting the primary antibody. Tissue sections were also double labeled using PEP5A2 and a polyclonal antibody against sucrase isomaltase (30) or a polyclonal antibody raised against the ectodomain of plgR (3). Samples were incubated with PEP5A2 as above, rinsed with PBS, and incubated for 30 min or 1 h at room temperature with the anti-sucrase isomaltase antibody or the anti-plgR antibody. Binding was detected with anti-species FITC- and Cy3-conjugated IgG antibodies. All specimens were examined under a Zeiss photomicroscope equipped with epifluorescence optics (Zeiss, Oberkochen, Germany) and photographed. Double-labeled specimens were also examined by confocal laser scanning microscopy (CLSM; Leica, Wetzlar, Germany). Tissue sections were viewed in the x-y plane, and the images were photographed.

**RESULTS**

CIC-5 mRNA and protein in tissues. RT-PCR experiments using a set of primers specific for the rat rCIC-5 (39) were performed to study the expression of the CIC-5 gene in the intestine and colon. Similar amounts of CIC-5 transcripts were detected in the rat duodenum, jejunum, ileum, proximal and distal colon, and kidney compared with the amount of hGAPDH transcripts, used as internal standard (Fig. 1). No CIC-5 mRNA was detected in skeletal muscle or in non-reverse-transcribed colon and kidney RNAs or by omitting cDNA (Fig. 1).

Western blot analyses to detect CIC-5 were then performed on membrane preparations from normal rat tissues using the PEP5A2 antibody. We have previously shown that this affinity-purified rabbit polyclonal antiserum is specific for CIC-5 and detects a protein band of the expected size (83-kDa) in the kidney and in rCIC-5-injected oocytes (15). The results from Western blot analyses using the same PEP5A2 antiserum revealed a single protein band of ~80-kDa in kidney cortex and jejunum membrane preparations (Fig. 2A). As controls, the expected CIC-5 83-kDa band was not detected in lung (15) or in jejunum by omitting the primary antibody (Fig. 2A). PEP5A2 detected a major 83-kDa band not only in the rat jejunum but also in duodenum, ileum, and proximal and distal colon (Fig. 2B, top). The duodenum and proximal and distal colon contained more CIC-5 protein than did the terminal ileum, using β-actin as internal standard (Fig. 2B, bottom). However, CIC-5 was less abundant in all intestinal tissues than in the rat kidney (Fig. 2B, top). As controls, no band was detected in these tissues when the primary PEP5A2 antibody was omitted (data not shown). These results indicate that both mRNA and protein of the CIC-5 Cl− channel are present all along the anterior-posterior axis of the intestinal tract.

Cellular distribution of CIC-5 in the intestine and colon. Indirect immunofluorescence was used to determine the cellular distribution of CIC-5 in frozen tissue sections of the rat intestine and colon. All sections from the duodenum, jejunum, ileum, and colon were stained with PEP5A2 (Fig. 3, A–D). The staining appeared to be mainly in the cytoplasm of epithelial intestinal cells, above nuclei, and to a lesser extent in the cytoplasm near the basal side of the cells (Fig. 3, A–C). The cytoplasm was stained more diffusely in colon cells...
Fig. 2. CIC-5 in normal tissues of adult rats. A: Western blots of crude membrane preparations (25 μg) from kidney (lane 1), jejunum (lane 2), and lung (lane 3) probed with the affinity-purified serum (PEP5A2) raised against the amino terminal peptide sequence of the rat CIC-5 PEP5A2 recognize a single band of ~80 kDa in kidney (lane 1) and jejunum (lane 2) but not in lung (lane 3) or jejunum when the primary antibody was omitted (lane 4). B: Western blotting using PEP5A2 (top) and an anti-β-actin antibody, used as internal standard (bottom), was also performed on membrane preparations from duodenum (lane 5), jejunum (lane 6), ileum (lane 7), proximal colon (lane 8), distal colon (lane 9), and kidney (lanes 10 and 11). For comparison, the amount of CIC-5 protein detected in all intestinal tissues (lanes 5–9: 50 μg) was quantitatively lower than in kidney membrane preparations (lane 10: 5 μg; lane 11: 25 μg).

(Fig. 3D). There appeared to be no clear differences in staining intensity along the crypt-villus axis of the intestine and colon walls (Fig. 3, A and D). The pattern of PEP5A2 immunostaining in frozen kidney sections of the same rats was identical to that previously reported by Günther et al. (15) using the same PEP5A2 antiserum. The region of the cytoplasm underlying the apical membrane of epithelial cells and some cells from the collecting duct, shown to correspond to intercalated cells (15), were positively stained (Fig. 3E). Like the control, no staining was observed when the primary antibody was omitted from kidney sections (data not shown) or colon sections (Fig. 3F). To further assess the specificity of the labeling provided by PEP5A2, indirect immunofluorescence was performed on jejunal sections using PEP5A2 without or with increasing amounts of the synthetic peptide against which it was raised (Fig. 4). The PEP5A2 labeling was specific, as it could be blocked by preincubation with increasing amounts of synthetic peptide (Fig. 4, A–D for comparison). Taken together, the immunostaining provided by PEP5A2 was specific and revealed the presence of CIC-5 in digestive tissues.

Subcellular distribution of CIC-5 in small intestine and colon cells. CLSM analyses of frozen sections of jejunum and colon stained with PEP5A2 showed a striking distribution of CIC-5 within the small intestine and colon cells (Fig. 5). The staining of enterocyte cytoplasm gradually increased from the region underlying the apical membrane to reach a maximum intensity in the cytoplasm just above the nucleus (Fig. 5A). The staining of the cytoplasm of colon cells was more diffuse (Fig. 5C). CLSM analyses of immunostained sections from jejunum and colon with PEP5A2 revealed a fine punctate staining in the cytoplasm, predominantly concentrated above the nuclei (Fig. 5, B and D), and a weak punctate cytoplasmic staining in the basal compartment of the cytoplasm of enterocytes and colon cells (Fig. 5, B and D).

Previous studies on rat and human kidneys have shown that CIC-5 is hardly detectable in the apical brush-border membrane of rat and human proximal tubule cells (9, 15). Therefore, we processed cryosections of jejunum for double indirect immunofluorescence using PEP5A2 and a polyclonal antibody against sucrase isomaltase, a specific hydrolase of the intestinal brush border (22). Enterocytes were immunostained for both sucrase isomaltase and CIC-5, but the two patterns were not strictly superimposed (Fig. 6, A and B). CLSM analysis revealed that the CIC-5 channel could not be colocalized with sucrase isomaltase. The punctate staining provided by the PEP5A2 antibody thus suggested that, like in renal proximal tubule cells (15), CIC-5 was concentrated in intracellular vesicular-like structures (Fig. 6C).

Subcellular distributions of CIC-5 and vacuolar H\(^+\)-ATPase, rab4, and rab5a. Previous studies have shown that CIC-5 is colocalized with vacuolar H\(^+\)-ATPase and rab5a in renal proximal tubule cells (9, 15, 24, 32). We therefore examined the subcellular distributions of CIC-5 and several organelle-specific markers in a jejunal cell preparation using differential centrifugation (9). This was done because indirect immunofluorescence studies using the available antibodies directed against the vascular proton ATPase and small GTPase rabs did not provide satisfactory results. Western blotting was performed on the plasma membrane-enriched fraction (fraction I), low-speed (fraction II) and high-speed (fraction III) pelleted fractions using an anti-E-cadherin antibody, PEP5A2, an anti-vacuolar H\(^+\)-ATPase antibody, and three different anti-rab antibodies (Fig. 7). E-cadherin, an adhesion molecule expressed in basolateral membranes of epithelial cells, was mainly found in fraction I and to a much lesser extent in fraction II and was almost not detectable in fraction III (Fig. 7). CIC-5 was mainly found in pelleted fractions II and III and was almost undetectable in the E-cadherin-enriched plasma membrane fraction I. The 70-kDa subunit of the vacuolar H\(^+\)-ATPase was also almost undetectable in fraction I but had almost the
same pattern of subcellular distribution as ClC-5 in pelleted fractions II and III (Fig. 7). Rab4, which is located in the early endosome and plasma membrane recycling pathway (8, 42), was almost equally distributed in fractions I, II, and III. Rab5a, which is found in the plasma membrane, clathrin-coated vesicles, and early endosomes (6, 7), was also detected in all three fractions, mainly in fraction II (Fig. 7). Rab6, a ubiquitous small GTPase associated with the membranes of the Golgi complex (1, 11), was mainly detected in plasma membrane-enriched fraction I and to a lesser extent in fraction II, with almost none in fraction III (Fig. 7). Although the separation of membrane fractions was not optimal, especially for the plasma membrane fraction I contaminated with Golgi membranes, these results clearly indicate that the ClC-5 Cl\(^{-}\) channel is mainly in the subcellular fractions that are highly enriched in rab4, rab5a, and H\(^{+}\)-ATPase. Both the punctate cytoplasmic immunostaining obtained with the PEP5A2 antibody (see Fig. 6C) and the detection of the ClC-5 Cl\(^{-}\) channel in subcellular fractions highly enriched in rab4 and rab5a involved in regulating the membrane traffic of the endocytotic pathway strongly suggest that ClC-5 is concentrated in vesicular organelles corresponding to early and/or recycling endosomes.
ClC-5 partially colocalizes with transcytosed pIgRs. The striking colocalization of ClC-5 with H\(^+\)-ATPase and endocytosed β₂-microglobulin in renal proximal tubule cells strongly suggested that this Cl\(^-\) channel plays an important role in the dissipation of the electrical gradient generated by the vacuolar H\(^+\)-ATPase in endosomes (15, 32). The similar distribution of ClC-5 in intestinal cells prompted us to test whether it was associated with endocytosed or transcytosed proteins via endosomal vesicles. Previous studies have demonstrated that pIgR, which can bind its ligand dIgA, represents a useful model system for studying transcytosis (2, 36). With or without dIgA, the pIgR is internalized from the basolateral plasma membrane and delivered to early endosomes (2). The pIgR then moves through several endosome compartments before reaching the apical plasma membrane. At this site, the extracellular binding domain of the pIgR is cleaved (2). This cleaved fragment, also called the secretory component (SC), is normally released with dIgA. We have previously shown the presence of pIgRs mainly in the cytoplasm from mouse intestinal crypt cells by using anti-pIgR antibodies (3). Cryosections of rat jejunum processed for indirect immunofluorescence using an antibody raised against the ectodomain of pIgR showed a similar distribution of pIgRs (Fig. 8A). Double indirect immunofluorescence with PEP5A2 and the anti-pIgR antibody analyzed by CLSM clearly showed that the endocytosed pIgRs detected in the region above the nuclei were colocalized with ClC-5 (Fig. 8, B–D). In contrast, the immunostaining provided by the anti-pIgR antibody, which was detected in the apical region beneath the intestinal brush border, presumably corresponding to cleaved SC released from vesicles, was
not associated with the ClC-5 Cl\textsuperscript{2} channel (Fig. 8E). Thus these results provided more direct evidence that ClC-5 is present in endosomes from the endocytotic and transcytotic pathways of intestinal cells.

**DISCUSSION**

The ClC-5 Cl\textsuperscript{2} channel is predominantly expressed in kidney epithelial cells and is probably involved in the endocytosis of low-molecular-weight proteins (9, 15). Previous mRNA and Western blot analyses also suggest that this Cl\textsuperscript{2} channel is present in other tissues, including the brain, testis, and liver (15, 39). We have now shown that the ClC-5 Cl\textsuperscript{2} channel is present in the epithelial cells lining the villi of the rat small intestine and colon. ClC-5 mRNA was detected by RT-PCR in all of the segments of the anteroposterior axis of the small intestine and the proximal and distal parts of the colon examined, and Western blot analyses revealed ClC-5 in these tissues. Few Cl\textsuperscript{2} channels have been identified in intestinal and colon cells. The cystic fibrosis transmembrane conductance regulator (CFTR) gene, belonging to the superfamily of ATP-binding cassette transporters, is expressed in crypt cells (40) and plays an important role in the cAMP-dependent regulated secretion of Cl\textsuperscript{2} (34). The ClC-2 Cl\textsuperscript{2} channel is also present in the mouse duodenal epithelium (21). The exact function of ClC-2 is still not clear. Its mRNA expression declines during late gestation (27) and is unaltered in mice with cystic fibrosis (21). These results indirectly suggest that ClC-2 has some housekeeping function.

ClC-3 is present in colonic myocytes (10), but it has not been shown to occur in colon and/or small intestinal epithelial cells. Recently, a potential human Ca\textsuperscript{2+}-sensitive Cl\textsuperscript{2} channel, and its murine counterpart, has been shown to be expressed in basal crypt epithelia and goblet cells (12, 13). Thus, to our knowledge, ClC-3 is the fourth potential Cl\textsuperscript{2} channel to be identified in intestinal epithelial cells. As in renal proximal tubule and intercalated collecting duct cells, the ClC-5 Cl\textsuperscript{2} channel in intestinal and colon epithelial cells is very predominantly, if not exclusively, intracellular.

ClC-5 has been shown to colocalize with the proton pump in renal proximal tubule and a-intercalated cells (9, 15, 32). The results from fractionation studies on jejunum cell preparations also indicate that ClC-5 and vacuolar H\textsuperscript{+}-ATPase are detected in both low- and high-speed pelleted subcellular fractions containing H\textsuperscript{+}-ATPase subunit H\textsuperscript{1}-ATPase subunit.
rab4 and rab5a, two ras-like GTP-binding proteins associated with early endosomes (7, 42). Consistent with the fact that the vacuolar-type H⁺-ATPase is absent from the brush-border membrane vesicles of enterocytes (31), neither the 70-kDa H⁺-ATPase subunit nor ClC-5 was clearly detectable in the plasma membrane fraction of jejunum cells. Thus the distribution of ClC-5 in intestinal cells closely resembles that of ClC-5 in renal epithelial tubule cells. The results from subcellular fractionated studies and immunohistochemical studies also strongly suggest that ClC-5 is predominantly, if not exclusively, located in densely packed endocytic vesicles. The intravesicular acidification of these vesicles mediated by vacuolar-type H⁺-ATPases is essential for various sorting processes (28). The parallel distributions of ClC-5, H⁺-ATPase, and rab GTPase proteins suggest that ClC-5 provides the electrical shunt necessary for the acidification of vesicles from the intestinal endocytic pathway (15).

The endocytic and transcytotic capacities of epithelial cells have been demonstrated in various cell systems (2, 16, 17, 36, 37). Interestingly, we found that the internalized pIgR, known to be delivered from basolateral endosomes to the apical endosomes in epithelial polarized cells (2, 36), is colocalized with ClC-5 in the intestinal secreting cells bordering the base of the villi. Thus these results provided further evidence that ClC-5 is expressed in some, but may be not all, of the endosomal compartments involved in the endocytosis and transcytosis of proteins.

It has also been suggested that ClC-5 plays a role in Ca²⁺ absorption. Patients suffering from Dent’s disease caused by inactivating mutations in ClC-5 have low-molecular-weight proteinuria, hypercalciuria, nephrocalcinosis, and nephrolithiasis (33). The primary mechanism by which hypercalciuria occurs remains a matter of debate. Sufferers from Dent’s disease often have low plasma parathyroid hormone concentrations and elevated plasma 1,25-dihydroxyvitamin D, a situation that contrasts with renal insufficiency of other origins (33). The hypercalciuria that occurs in Dent’s disease could result from abnormal regulation of vitamin D in the kidney or from the hyperabsorption of Ca²⁺ by the gut. Luyczkx et al. (25) have recently shown that transgenic mice with reduced ClC-5 expression are hypercalciuric when fed a normal Ca²⁺ diet but do not exhibit other altered renal functions. These authors also mention that ClC-5 is expressed throughout the mouse intestinal epithelium and hypothesize that ClC-5 regulates the absorption of Ca²⁺ across the gut mucosa. However, we did not detect ClC-5 at the apical brush-border membrane of intestinal cells. Thus it seems unlikely that ClC-5 is directly involved in the apical intestinal absorption of Ca²⁺. Furthermore, the primary cause of abnormal Ca²⁺ transport and regulation in Dent’s disease remains to be determined.

In summary, we have demonstrated the presence of the ClC-5 Cl⁻ channel in the small intestine and colon cells of the rat. Our data also suggest that this CLC channel, which is exclusively intracellular, is involved in some of the endocytic processes occurring in intestinal cells.

We thank Drs. D. K. Stone, R. Riba, B. Couray, and M. Kedinger for generously providing us with valuable antibodies. We thank E. Pringault for stimulating discussions. We also thank S. Roger and P. Didsier for photographic work and Dr. O. Parkes for editing assistance.

This study was supported by Institut National de la Santé et de la Recherche Médicale (INSERM). Dr. K.-C. Peng holds an INSERM postdoctoral (Poste vert) fellowship supported by the Conseil Régional de l’Ile de France.

REFERENCES


5. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas- 


9. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


13. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


17. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


21. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


25. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


29. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


33. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


37. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


41. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


45. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-


49. Murray CB, Chu S, and Zeitling PL. Am J Physiol Gas-

