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Authors

Talbot, Colleen

Lytle, Christian

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Am J Physiol Cell Physiol, February, 2011; 300 (2): C276-C286.

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Segregation of Na/H exchanger-3 and Cl/HCO₃ exchanger SLC26A3 (DRA) in rodent cecum and colon

Colleen Talbot¹ and Christian Lytle²

¹California State University, San Bernardino; and ²Division of Biomedical Sciences, University of California, Riverside, California

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Talbot C, Lytle C. Segregation of Na/H exchanger-3 and Cl/HCO₃ exchanger SLC26A3 (DRA) in rodent cecum and colon. *Am J Physiol Gastrointest Liver Physiol* 299: G358–G367, 2010. First published May 13, 2010; doi:10.1152/ajpgi.00151.2010.—The colon is believed to absorb NaCl via the coupled operation of apical Na/H exchanger-3 (NHE3) and Cl/HCO₃ exchanger SLC26A3 (DRA). Efficient coupling requires that NHE3 and DRA operate in close proximity within common luminal and cytosolic microenvironments. Thus we examined whether these proteins coexist along the apical margin of surface enterocytes by quantitative immunofluorescence microscopy in consecutive colon segments from nonfasted mice and rats. The cecocolonic profiles of NHE3 and DRA expression were roughly inverse; NHE3 was highest in proximal colon (PC) and negligible in distal colon, whereas DRA was absent in early PC and highest in the late midcolon, and DRA was prominent in the cecum whereas NHE3 was not. NHE3 and DRA coexisted only in the middle third of the colon. The consequences of unpaired NHE3/DRA expression on mucosal surface (subscript MS) pH and Na⁺ concentration ([Na⁺]) were assessed in nonfasted rats in situ using miniature electrodes. In the cecum, where only DRA is expressed, pH_{MS} was ~7.5, markedly higher than underlying stool (6.3), consistent with net HCO₃[−] secretion. In the early PC, where NHE3 is not expressed with DRA, pH_{MS} was acidic (6.2), consistent with unopposed H⁺ secretion. [Na⁺]_{MS} was ~60 mM in the cecum, decreased along the PC to ~20 mM, and declined further to ~10 mM distally. Cl[−] was secreted into the PC, then reabsorbed distally. Our results suggest a model in which 1) unpaired DRA activity in the cecum maintains an alkaline mucosal surface that could neutralize fermentative H⁺; 2) unpaired NHE3 activity in the early PC preserves an acidic mucosal surface that could energize short-chain fatty acid absorption; and 3) coupled NHE3/DRA activities in the midcolon allow for vigorous NaCl absorption at a neutral pH_{MS}.

mouse; rat; intestine; electroneutral NaCl absorption

TRANSPORT ACROSS THE COLONIC epithelium allows for efficient salvage of water and solutes [Na⁺, Cl[−], short-chain fatty acid (SCFA)], maintenance of the resident microbial ecosystem, and optimal fecal consistency. The absorptive movements of Na⁺ and Cl[−] in the mammalian colon are, to a large degree, mutually interdependent, electrically neutral, and associated with secretory movements of H⁺ and HCO₃[−] (28, 29, 35, 57, 71). It is generally accepted that electroneutral NaCl absorption by the intestine largely reflects the tandem operation of apical Na/H exchange and Cl/HCO₃ exchange processes, and studies of rodent colon have established that these processes primarily involve the Na/H exchanger NHE3 (SLC9A3) and the Cl/HCO₃ exchanger SLC26A3 (alias downregulated in adenoma, or DRA) (5, 7, 57, 71, 86).

Both NHE3 and DRA are prominently expressed in colonic epithelial cells as they migrate out of the crypt and acquire their differentiated absorptive phenotype (13, 18, 44, 53, 67, 74). In mouse models, genetic ablation of NHE3 results in severe intestinal fluid accumulation with subsequent Na⁺ diarrhea, net base loss, and metabolic acidosis (10, 54, 73). Another apical Na/H exchanger, NHE2 (SLC9A2), is found in both surface and crypt epithelial cells (2, 8, 9, 18, 25, 36) where it has been implicated in intracellular pH and volume regulation (2). The absence of overt absorptive dysfunction in NHE2 knockout mice, or aggravation of diarrhea by additional disruption of NHE2 in NHE3 knockout mice (54), suggests that NHE2 plays a less important role in intestinal NaCl absorption.

Several lines of evidence have indicated that DRA is a major apical Cl/HCO₃ exchanger and an essential participant in colonic NaCl absorption (60–62, 74). In rodent intestine, mRNA encoding DRA is most abundant in the cecum and distal colon (3, 77), with lower amounts in the proximal colon (3, 60). Similarly, in human intestine, DRA transcript is detected at high levels in the appendix and distal colon (39). The central role of DRA in intestinal Cl[−] absorption and HCO₃[−] secretion is demonstrated by the hereditary disease congenital chloride-losing diarrhea (CLD), an autosomal recessive disorder characterized by lifelong diarrhea (acidic and salty), hypovolemia, and metabolic alkalosis (39, 40, 48, 61, 74). Patients with CLD lack a Cl/HCO₃ exchange activity that is normally present in the colon and distal ileum (41, 45, 68) and have specific mutations in the DRA gene (39). The pathological presentation of CLD is largely recapitulated in DRA knockout mice (74), and colonic mucosa isolated from these mice is deficient in both HCO₃[−]-coupled Cl[−] absorption (74) and Cl[−]-coupled HCO₃[−] secretion (79). In addition to bidirectional Cl/HCO₃ exchange, DRA exhibits some capacity to trade Cl[−] for NO₃[−] (37) or oxalate[−] (17) and therefore might function more broadly in intestinal anion transport (74). Another apical anion exchanger, SLC26A6 (alias PAT1), is prominently expressed in the proximal small intestine but not in the colon and is therefore unable to cooperate with, or substitute for, DRA in colonic anion transport (87).

The NHE3 and DRA transport processes are presumed to be coupled through the reversible interconversion of their substrates (H⁺ and HCO₃[−]) and membrane-permeable products (CO₂ and H₂O). Thermodynamic coupling is promoted by the enzymatic catalysis of this interconversion by cytoplasmic (CA-II) and extracellular (CA-IV) forms of carbonic anhydrase (CA) (15, 34, 78). Kinetic coupling is afforded by reciprocal allosteric regulation of NHE3 (38) and DRA (17, 37) by cytosolic pH. Both of these coupling mechanisms require that NHE3 and DRA operate in close proximity within common luminal and cytosolic microenvironments. Although there is

Address for reprint requests and other correspondence: C. Lytle, Division of Biomedical Sciences, Univ. of California, Riverside, CA 92521-0121 (e-mail: christian.lytle@ucr.edu).

evidence that DRA and NHE3 can physically combine through interactions with PDZ adaptor proteins that modulate their transport activities (52), whether both transporters actually coexist along the absorptive surface of the colon, and in which regions of the colon, has not been explored. The concept that the two exchangers are operationally coupled, thermodynamically and allosterically, by local changes in pH or HCO_3^- (16, 20, 50, 62, 81) is consistent with evidence that colonic NaCl absorption is acutely sensitive to changes in HCO_3^- and CO_2 concentrations (15, 16, 35, 41) and depends on carbonic anhydrase activity (15). Coupling of apical Na/H and Cl/base exchange activities has been demonstrated in cultured epithelial cells exogenously expressing NHE3 and DRA (62) and in the distal half of the rat colon (34).

Although it is well established that the rates and characteristics of Na^+ and Cl^- transport differ markedly between the proximal and distal segments of the colon (29, 71), the spatial profile of electroneutral NaCl absorption along the cecocolonic axis remains poorly defined. To better understand the anatomical and functional compartmentation of the colon, and to reassess the applicability of the NHE3/DRA coupling concept in colonic electrolyte absorption, we determined the expression profiles of apical NHE3 and DRA proteins in the cecum and colon of rats and mice. These profiles were compared with mucosal surface pH and Na^+ concentration ($[\text{Na}^+]$), and with stool water, Na^+ , and Cl^- contents in anesthetized rats. We discovered that NHE3 and DRA do not coexist uniformly along the cecocolonic axis. In the proximal colon, NHE3 is prominently expressed without DRA, and the mucosal surface pH was found to be uniquely acidic (~ 6.2), possibly reflecting net H^+ secretion via unpaired Na/H exchange. By contrast, in the cecum and distal colon, DRA is strongly expressed without NHE3, and here the mucosal surface pH was found to be slightly alkaline (~ 7.5), consistent with net HCO_3^- secretion via unpaired Cl/ HCO_3^- exchange. The conventional concept of efficient functional coupling between colonic NHE3 and DRA seems possible only within the middle third of the rodent colon, where both exchangers coexist in the same absorptive microdomain.

METHODS

Animals. Female CD1 mice (8–12 wk old) and Sprague-Dawley rats (190–270 g) were purchased from Charles River Laboratories. Animals were housed at $23 \pm 1^\circ\text{C}$ on a 12:12-h light-dark cycle and allowed free access to water and a standard rodent diet before use. In situ measurements of intestinal surface pH and $[\text{Na}^+]$ were carried out on anesthetized rats. All animals were euthanized by CO_2 asphyxiation. Animal protocols were approved by the University of California, Riverside, Institutional Animal Care and Use Committee.

Abundance of apical NHE3 and DRA proteins in surface colonocytes. The density profile of NHE3 and DRA immunolabel along the apical margin of surface enterocytes was determined by quantitative confocal fluorescence microscopy. A series of 8–12 short (~ 1 cm) segments of rat and mouse intestine extending from the ileocecal junction to the anus were fixed in ice-cold neutral buffered formalin for 5 h, rinsed thoroughly with PBS, and infiltrated overnight with cryoprotectant (30% sucrose in PBS). Tissues were placed in OTC medium (Triangle Biomedical Sciences) and frozen between copper blocks on dry ice. Serial $10\text{ }\mu\text{m}$ cross sections of the intestinal wall, with the crypts in longitudinal profile, were cut from the midpoint of the intestinal segment and thaw mounted onto a glass slide coated with poly-L-lysine (0.1 mg/ml, Sigma P1524). Rehy-

drated sections were treated with 1% SDS in PBS for 5 min to unmask antigenic sites, then incubated sequentially with blocking solution (PBS containing 5% goat serum, 0.05% Tween-20, 0.1% glycine, and 3 mM EDTA, pH 7.4), primary antibody (overnight at 4°C), secondary antibody conjugated to Alexa Fluor-488 (Molecular Probes), and the nuclear stain ToPro-3 (Molecular Probes). We employed well-characterized rabbit polyclonal antibodies directed against rat NHE3 (NHE3-C00) (88) provided by Dr. Alicia McDonough (University of Southern California-Keck School of Medicine) and DRA-Pab-I20F against the carboxy terminus of DRA (13, 74) kindly provided by Dr. Clifford Schweinfest (Medical University of South Carolina).

Images were acquired with a Zeiss LSM-510 confocal microscope and $\times 25$ water immersion (0.8 NA Plan Neofluar) or $\times 40$ oil immersion (1.30 Plan Neofluar) objectives. After determining the full range of fluorescence intensities within a given experimental series, we adjusted acquisition parameters to ensure that all signals were recorded within the linear range of the detector. For each intestinal segment, three to six representative digital images (8 bits/channel, $1,024 \times 1,024$ pixels) were acquired at $\times 0.7$ zoom with a $90\text{ }\mu\text{m}$ pinhole to obtain a $521 \times 521\text{ }\mu\text{m}$ field across an optical slice of $2\text{ }\mu\text{m}$ depth. Image files were imported into MCID Elite software (Imaging Research, St. Catharines, Ontario, Canada) for quantitation of regional fluorescence. The apical margin of contiguous surface enterocytes was carefully traced by use of a ribbon-shaped cursor centered on the apical fluorescence (Fig. 1). The thickness of the ribbon was set to the width of the enterocyte nucleus ($\sim 7\text{ }\mu\text{m}$). The selected area, which included the apical plasma membrane and a subapical zone $\sim 3\text{ }\mu\text{m}$ inside the cell, was considered the apical compartment where functional (plasma membrane) and reserve (subapical recycling endosome) transport units are expected to reside and traffic (4, 24). To correct for nonspecific fluorescence, readings of cells within the lamina propria were averaged and subtracted from all other readings; these background corrections amounted to no more than 10% of the maximal specific NHE3 and DRA signals. Data from 3–10 regions in each image were averaged, and three to six images of each intestinal segment were further averaged to obtain the expression profile for a particular animal. Finally, data from three to six animals were plotted against relative axial position expressed as % length, with the cecocolical junction designated 0% and the anus 100% (see Fig. 3). We define here three segments of the colon based on their distinctive patterns of transporter expression: proximal (0–20% length), mid (20–60% length), and distal (60–100% length).

Fig. 1. Immunodetection of apical Na/H exchanger-3 (NHE3; top) and Cl/ HCO_3^- exchanger SLC26A3 (DRA; bottom) proteins in rat midcolon enterocytes. Immunofluorescence intensities along the apical margin of surface enterocytes were quantified by digital image analysis of mucosal cross sections by using a $7\text{-}\mu\text{m}$ -wide ribbon (region of interest), outlined in red. Nuclei are labeled blue with ToPro-3 dye. A single crypt is labeled *.

Mucosal surface pH and $[Na^+]$ profiles along the distal intestine. The pH and $[Na^+]$ of the mucosal surface and luminal contents of the cecum and colon were measured in anesthetized rats by using flexible esophageal-type miniature electrodes (MI-506 and MI-420, Microelectrodes, Bedford, NH). These electrodes have small (1.3-mm-diameter) round glass bulb tips with smooth surfaces to minimize tissue injury. Rats were anesthetized by intraperitoneal injection of 22 mg/kg ketamine, 5 mg/kg xylazine, and 0.75 mg/kg acepromazine and placed in dorsal recumbency on a thermoregulated (39°C) pad. All procedures were completed within ~20 min, and throughout this interval, breathing remained normal and unlabored without tracheostomy or mechanical ventilation. Short segments of terminal ileum, cecum, proximal and midcolon were exteriorized through a 2-cm incision in the upper left quadrant of the abdominal wall, using care to avoid damaging blood vessels, and kept moist with gauze soaked with prewarmed isotonic mannitol. Electrodes were inserted through small (~3 mm) antimesenteric incisions in the bowel wall and positioned parallel to the mucosal surface. During recordings, the area of mucosal surface in contact with the ion-sensitive glass bulb was maximized by applying gentle outward tension to the electrode to form a shallow pocket of the intestinal wall. Readings were taken at two to three sites of a measured distance proximal to, and then distal to, the incision, and held in place at each site until stable readings were recorded (~10 s). For access to the distal colon, electrodes were inserted via the anus stepwise at 1-cm intervals. A miniature Ag-AgCl electrode (Microelectrodes, MI-402), inserted alongside the ion-sensing electrode, was used as the reference. The two electrode tips were juxtaposed against the mucosal surface no more than 1 cm apart to minimize the influence of longitudinally variable intraluminal potentials. Similar results were obtained in a subset of experiments by using a miniature glass pH combination electrode with a 0.75-mm-diameter tip and internal reference (MI-415, Microelectrodes). In some experiments, the external reference electrode was anchored subcutaneously in the inguinal region with a suture, without correction for transmucosal potential differences (6–12 mV) (30), to allow comparison to previous data obtained in the same manner (56). Electrodes were connected to a pH meter (Orion 720A) and calibrated against phosphate pH buffer standards (4.0–10.0) or $[Na^+]$ standards (0–100 mM in 20 mM K-acetate). The rate and linearity of the electrode responses were checked before and after each experiment. The high selectivity of the Na^+ electrode for Na^+ over K^+ (1,000:1) and NH_4^+ (3,000:1) made correction for these ions in stool water unnecessary. Afterward, the distal intestine was isolated, and the recording locations were confirmed.

Fecal water, Na^+ , and Cl^- . The distal intestine (terminal ileum to anus) was removed from a euthanized rat, placed on an ice-cold tray with gentle stretching, and trimmed of mesentery. After their relative locations were recorded, stool samples (50–200 mg) were removed through small incisions in the intestinal wall and immediately sealed in prebaked, preweighed microfuge tubes. Each tube was weighed, then uncapped and dried at 60°C for 24 h. After reweighing, the residue was extracted in distilled water (5-fold volume/dry wt) for >24 h. After mixing and centrifugation, $[Na^+]$ in the supernatant was measured via a Na^+ electrode (MI-420, Microelectrodes) or a flame photometer (Beckman Kline Flame). In a subset of samples that had sufficient volume, Cl^- concentration ($[Cl^-]$) was measured by coulometric titration via a Buchler-Cotlove chloridometer. Water content was normalized to dry stool weight (μ l/mg dry wt) and plotted against the sample's origin, expressed as % length, with the cecocolic junction designated 0% and the anus 100%. Stool ion concentrations were calculated as the ratio of ion content and water content of the same fecal sample.

Western blot analysis. The abundance of NHE3 and DRA protein in mucosa-submucosa preparations from colonic segments was measured by Western blot analysis by a standard procedure, as described previously (58). The intestinal wall was pinned mucosal-side down on an ice-cold plate coated with Sylgard, cut open along the mesenteric

border, and stripped of serosal and muscle layers by blunt dissection using the edge of a glass slide. The tissue was snap frozen, then thawed in an ice-cold lysis solution containing 3 mM HEPES (pH 7.4), 3 mM $MgSO_4$, 2 mM EGTA, and protease inhibitors. The insoluble fraction was extracted by brief sonication in a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 1% deoxycholate, 0.5% Triton X-100, 0.1% SDS, and protease inhibitors. Samples of clarified supernatant, each containing 50 μ g protein, were separated by SDS-PAGE, transferred to polyvinylidene difluoride, and probed with DRA-PAb-I20F or NHE3-C00 antibodies.

Data are presented as means $\pm$ SE. Statistical significance between stool water contents and ion concentrations was calculated by ANOVA where the data was binned into four discrete functional categories: cecum, proximal colon (0–20% colon length), midcolon (20–60%), and distal colon (60–100% colon length). The relative percent location was arcsine transformed before the analysis. Multivariate analysis was used to determine which functional regions produced significant differences in fecal water and ion concentrations. $P < 0.05$ was considered statistically significant.

RESULTS

To evaluate the premise that neutral NaCl absorption by the colon reflects the coupled operation of NHE3 and DRA, we examined whether the two transport proteins coexist along the absorptive microdomain (apical membrane) of surface enterocytes by immunofluorescence microscopy. In the rat midcolon, antibodies selective for NHE3 and DRA prominently labeled only the apical margin of the differentiated colonocytes that inhabit the mucosal surface and the upper third of the crypts (Fig. 1), in accord with previous reports (36, 42, 44, 74). However, qualitative comparison of tissues obtained from different regions of colon and cecum revealed contrasting profiles of NHE3 and DRA expression (Fig. 2). In the proximal colon, NHE3 was prominently expressed without DRA, whereas in the cecum and distal colon DRA was strongly expressed without NHE3. The only region with both exchangers was the midcolon.

To obtain a more precise map of apical NHE3 and DRA protein abundance along the colonic axis, a larger series of tissue sections was cut from consecutive intestinal segments and immunolabeled under identical conditions. Digital image analysis software was used to quantify the immunofluorescence (pixel intensity) along the apical margin of surface colonocytes, delimited by tracing a ribbon along the luminal margin and crypt vestibule, with interruptions to exclude cells deep within the crypt annulus, as described in METHODS and illustrated in Fig. 1. A ribbon width of 7 μ m was chosen so as to fully encompass the microvillus brush border (0.7–1.3 μ m) and the apical third of surface epithelial cells where both functional (plasma membrane) and reserve (subapical vesicle) forms of NHE3 (24) and DRA (33, 37) are expected to reside and traffic. The aggregate data (Fig. 3) confirmed that NHE3 and DRA do not substantially coexist in the cecum, early proximal colon, or distal colon of either the mouse or rat. In both rodent models, the absorptive microdomain of the cecum and distal colon (60–100% length) contained abundant DRA yet little or no NHE3, whereas the early proximal colon (0–20% length) contained only NHE3. The only segment in which NHE3 and DRA proteins appreciably coexisted in the apical membrane was the midcolon (20–60% length). These results are consistent with previous evidence that DRA mRNA is most abundant in the rat cecum and distal colon, with low

Fig. 2. Regional expression of NHE3 (*top*) and DRA (*bottom*) along the rat colon and cecum. Tissues isolated from 8 regions the distal intestine were immunolabeled and imaged under identical conditions, with fluorescence recorded within the linear range of the detector. Nuclei are labeled blue with ToPro-3 dye. The origin of each tissue along the colonic axis, expressed as % colon length, is indicated with an arrow.

levels in the proximal colon (3). In a more detailed study of the mouse cecum, we found DRA at uniformly high levels in the apex, corpus, and ampulla; in contrast, contiguous apical NHE3 labeling was apparent only in the blind end (apex) of the mouse cecum, although at low levels (Fig. 3). In other regions of the cecum, small patches of apical NHE3 labeling were sporadically observed; we presume these to be rare “brush cells” (63) whose contribution to cecal function remains uncertain.

Comparable distributions of NHE3 and DRA were observed in Western blots of protein extracted from mucosal scrapings of rat intestinal segments (Fig. 4). The NHE3-C00 antibody prominently detected a single band (~ 80 kDa) that was present in the proximal colon (0–20% colon length) but not in either the cecum or distal colon. As reported in previous studies of DRA of mice (74) and rats (44), the DRA-PAB-I20F antibody detected a single protein of ~ 100 kDa. This DRA band was abundant in the cecum (not shown) and distal colon (60–85% colon length) yet undetectable in the proximal colon (0–20%); lesser amounts of DRA were found in the early midcolon (30–55%) and extreme distal segment (90–100%). This distribution of DRA protein is consistent with previous data on DRA mRNA along the rodent intestine (3, 60, 77). Similar Western blot results were obtained with mucosal tissues isolated from intestinal segments of mice (data not shown).

Our finding that NHE3 is not paired with DRA in the proximal colon, and that DRA is not paired with NHE3 in the cecum and distal colon, has important functional implications. Without alternative mechanisms for H^+ gradient coupling, the epithelial surface would be rendered more acidic by NHE3 activity in the proximal colon and more alkaline by DRA activity in the cecum and distal colon, as illustrated in Fig. 6. To test these predictions, we measured the pH at the mucosal surface (pH_{ms}) in nonfasted anesthetized rats using a flexible

esophageal-type miniature glass electrode. When the electrode tip was placed in the ileum, ~ 2 cm from an incision at the ileocecal junction, it recorded an average pH_{ms} of 7.65 ± 0.25 (Fig. 5A). In the midcecum (corpus), the electrode registered a pH_{ms} of 7.45 ± 0.18 , not significantly different from the ileum and in agreement with previous measurements (56). After the electrode tip was moved ~ 3 mm into the cecal stool, a much lower pH_{ms} (6.26 ± 0.13) was recorded, corroborating previous measurements of freshly isolated rat cecal stool (pH 6.42 ± 0.13 ; Ref. 65). As the electrode was gently advanced beyond the ileocecal junction, pH_{ms} decreased abruptly to ~ 6.2 and remained acidic throughout the proximal colon (Fig. 5A). Unlike the cecum, where a steep juxtamucosal pH gradient was evident, the mucosal surface and the underlying stool were equally acidic in the proximal colon (data not shown). Just centimeters downstream, after 20% colon length, pH_{ms} increased sharply and remained alkaline ($pH \sim 7.5$) throughout the mid and distal colon (40–90% colon). Similar juxtamucosal pH profiles along the cecocolonic axis of mice have been reported (46). Our results are consistent with recent evidence that the cecum and distal colon, but not the proximal colon, secrete net base equivalents (46). It is evident that the cecocolonic profiles of pH_{ms} (Fig. 5A) and apical DRA (Fig. 3) are remarkably similar; thus the mucosal surface proved to be alkaline in regions that express DRA and acidic only in a region of the proximal colon that expresses NHE3 without DRA.

After each pH measurement, the electrode was withdrawn and replaced with a Na^+ -sensing electrode of similar tip geometry. Electrode recordings indicated that $[Na^+]$ at the mucosal surface ($[Na^+]_{ms}$) was lower in the cecum (60 ± 13 mM) than in the terminal ileum (87 ± 3 mM), whereas measurements of volatile mass detected no difference in stool water content (Fig. 5B). Thus cecal epithelial cells must ac-

Fig. 3. Regional expression of apical NHE3 and DRA in rat (*left*) and mouse (*right*) colon and cecum (shaded bar). Immunofluorescence intensities are expressed as a percentage of the maximum observed within an experimental series. Different symbols represent data from different animals expressed as means $\pm$ SD, and each symbol represents the average observed in 10–60 images. In 1 mouse, apical NHE3 and DRA abundance was quantified in 3 regions of the cecum: apex (Ap), midcorpus (C), and ampulla (A).

tively absorb Na^+ by a mechanism that does not involve NHE3. Electrode recordings also indicated that $[\text{Na}^+]_{\text{ms}}$ decreases sharply along the proximal colon and continues to decrease along the early midcolon (Fig. 5C). On transit through these segments, the stool lost approximately half of its water

Fig. 4. Western blot analysis of total mucosal NHE3 and DRA proteins in rat colon and cecum. Mucosal scrapings were isolated from the midcecum and various regions of the colon and blots containing equivalent amounts of total tissue protein were probed for NHE3 (*top*) and DRA (*bottom*). Protein samples for NHE3 and DRA blots were isolated from different sets of rats.

(Fig. 5B), presumably through absorptive transport processes and osmosis. This was accompanied by a large drop in stool $[\text{Na}^+]$ (Fig. 5C). Further removal of Na^+ and water by the distal half of the colon was minimal. The conventional notion that Na^+ absorption is a major osmotic determinant of colonic water absorption is consistent with our observation of an approximate proportionality between stool Na^+ content and stool water content (112 data points; $R = 0.80$). Assuming that the absorbed fluid is approximately isosmotic to stool water ($332 \pm 15 \text{ mosM}$, $n = 3$) and plasma ($304 \pm 6 \text{ mosM}$, $n = 3$), Na^+ along with obligatory counteranions (mainly acetate $^-$, propionate $^-$, butyrate $^-$, and Cl^-) would account for approximately half of the total osmolyte absorbed by the rat colon. Measurements of bulk stool $[\text{Na}^+]$, based on the Na^+ and water contents of isolated stool, yielded values indistinguishable from $[\text{Na}^+]_{\text{ms}}$ everywhere except in the early proximal colon, where $[\text{Na}^+]_{\text{ms}}$ was slightly (yet not statistically) higher (Fig. 5C).

Paired measurements of Cl^- content and water in rat stool revealed Cl^- concentrations of 15–20 mM in the terminal ileum and cecum, in agreement with previous measurements (23). On transit of stool through the proximal colon (0–20% length), both the concentration (Fig. 5D) and amount of Cl^- in stool increased markedly. This observation, along with the tendency of stool to gain water within the early proximal colon (Fig. 5B), indicates that this segment carries out net Cl^-

Fig. 5. Mucosal surface and stool ion concentrations along the cecocolonic axis in anesthetized nonfasted rats. *A*: mucosal surface pH recorded with a miniature electrode. *B*: water content of stool samples isolated from the terminal ileum, midcecum, or different regions of colon. *C*: Na⁺ concentration ([Na⁺]) at the mucosal surface recorded with a miniature electrode (●) or in bulk stool measured by ion and water content analysis (○); means ± SD. *D*: Cl⁻ concentration ([Cl⁻]) in stool samples measured by ion and water content analysis; aggregate data from 3 rats.

secretion. As stool advanced through the early midcolon (20–40% colon length), most of its original Cl⁻, along with the extra amount contributed by the proximal colon, was reabsorbed. The axial loss of stool Cl⁻ was greatest over the colonic segment in which NHE3 is high and DRA expression increases abruptly (20–40% colon length). On exiting this segment, stool water contained no more than 10 mM Cl⁻, and further losses of Cl⁻ along the distal colon were minimal.

DISCUSSION

The absorptive functions of the large intestine depend critically on the spatial distribution and functional cooperation of numerous ion transporters. We have mapped the axial distribution of two apical transport proteins known to contribute importantly to colonic ion and water absorption. The reciprocal profiles of NHE3 and DRA expression observed along the colonic axis suggest the existence of three functionally distinct segments: proximal colon (0–20% length), midcolon (20–60% length), and distal colon (60–100% length). We propose a topographical model (Fig. 6) that provides a mechanistic basis for segmental variations in mucosal surface pH, stool [Na⁺], and stool [Cl⁻], and for earlier observations that electrolyte transport in these regions differs.

Cecum. The cecum functions as an anaerobic bioreactor in which a diverse microbial community consumes, stores, and

redistributes energy harvested from otherwise indigestible polysaccharides. The pronounced acidity of cecal stool (pH ≤ 6.3) has been shown to reflect the fermentative generation of SCFAs by resident bacteria (14, 22). In cecal stool, SCFAs like acetate, propionate, and butyrate accumulate to high concentrations (60, 25, and 15 mM, respectively) (11, 75, 80), and CO₂ reaches extreme levels (~350 mmHg) (69). Despite prodigious proton generation within the stool matrix, the adjacent mucosal surface maintains a remarkably stable alkaline (pH 7.5) microclimate. Previous studies using miniature electrodes or membrane-anchored reporters have established that an ionic microclimate, a stratum of fluid that maintains a composition distinct from the bulk luminal solution, exists along the intestinal surface (19, 32, 70). This microclimate is presumed to reflect a convection-restricted space that is the proximate source of, or sink for, transported ions and therefore should reflect, more or less, apical membrane transport activity. Because the thickness of this microclimate is uncertain, the extent to which our electrodes report on it, as opposed to the macroscopic juxtamucosal surface, is open to question. Nonetheless, the spatial resolution of our miniature electrodes, with a tip diameter of 1.3 mm, can clearly distinguish a pH at the surface of the cecum that is far less acidic than the adjacent stool. By contrast, no such juxtamucosal gradient was apparent for Na⁺. In addition to energetically limiting the extent of

Fig. 6. Schematic representation of the observed distributions and proposed activities of NHE3 and DRA along the rodent cecocolonic axis. Four distinct functional segments are distinguished on the basis of the regional expression of each transporter: cecum; proximal (Prox) colon (0–20% length); midcolon (20–60% length); distal colon (60–100% length). In the cecum, DRA functions without NHE3 to secrete HCO_3^- in defense against microbially generated acid. In the proximal colon, NHE3 activity preserves an acidic microdomain along the mucosal surface and thereby drives short-chain fatty acid (SCFA^-) absorption via apical $\text{SCFA}^-/\text{HCO}_3^-$ exchange and nonionic diffusion of protonated SCFA . In the midcolon, NHE3 and DRA coexist in the apical membrane of the same surface epithelial cells, allowing for efficient thermodynamic and regulatory coupling via cytosolic pH, in accord with the conventional view. In the distal colon, as in the cecum, DRA operates without NHE3, possibly to preserve an alkaline surface microclimate.

$\text{Cl}^-/\text{HCO}_3^-$ exchange, the existence of a stable alkaline layer along the surface of the cecum and distal colon is expected to reduce the proportion of unprotonated SCFAs available for apical transit via nonionic diffusion (12). On the basis of studies of isolated mouse cecum, this alkaline layer appears attributable to an apical $\text{SCFA}/\text{HCO}_3^-$ exchange process that is independent of MCT1 (47) and an apical Cl/HCO_3^- exchange process that is defective in DRA knockout mice (46, 79). In the rodent cecum, $\text{SCFA}/\text{HCO}_3^-$ exchange and Cl/HCO_3^- exchange are evidently not coupled to NHE3 or NHE2 activities, as they are in the midcolon (6), since Na^+ absorptive capacity is relatively low (72) and NHE3 protein expression is negligible (Figs. 3 and 4). Consequently, both anion exchange processes in the cecum appear optimized for efficient HCO_3^- secretion.

The secretion of HCO_3^- into the adherent mucus layer could protect the cecal mucosa from the prodigious load of luminal protons arising from bacterial fermentation and NH_3 absorption (47), by analogy to the familiar gastric mucus- HCO_3^- barrier model (1). Of interest is whether cecal HCO_3^- secretion appreciably influences bulk luminal pH. Studies employing anaerobic continuous-flow fermentors to model the intestinal microbiota have indicated that even modest shifts in pH, from 5.5 to 6.5, can profoundly influence the makeup and metabolic activities of this microbial ecosystem (85). Thus, in addition to its postulated role in mucosal protection, cecal HCO_3^- secretion may control bulk stool pH and, by doing so, regulate bacterial growth and fermentation patterns.

Proximal colon (0–20% colon length). In the rodent, as in the human, the proximal segment of the colon appears to have

the greatest capacity for Na^+ and water absorption per unit area, and much of this is probably associated with SCFA^- absorption (75). Our finding that the proximal colon expresses abundant NHE3 without DRA is consistent with functional evidence that, in the absence of SCFA^- , this segment absorbs Na^+ rapidly via Na/H exchange without transporting significant quantities of Cl^- or HCO_3^- (29, 46, 57). Another unique feature of the proximal colon is an acidic mucosal surface (rat: pH 6.2; mouse: pH 6.6) (46). We attribute this acidity to the strategic absence of Cl/HCO_3^- exchange and to the localized presence of NHE3-mediated Na/H exchange (Fig. 6, “Prox colon”). This acidic milieu could be utilized to sustain SCFA^- absorption via both apical $\text{SCFA}/\text{HCO}_3^-$ exchange (75, 82) and apical diffusion of uncharged (protonated) SCFAs (pK_a values ~ 4.8). In addition to coupling Na^+ and SCFA^- absorption, the perpetuation of stool acidity along the proximal colon would tend to favor the selective growth and fermentative activities of butyrate-producing bacterial species in this segment (85). The presence of NHE3 in a region of luminal acidity (pH ~ 6.2) is noteworthy given that the turnover rate of this isoform, measured as ^{22}Na influx, appears to be substantially inhibited (80%) when extracellular pH is this acidic (64). This effect, together with the thermodynamic constraint of luminal acidity, could limit, in a feedback manner, luminal acidification by Na/H exchange.

We were surprised to find that a considerable amount of Cl^- is added to stool in the early proximal colon and then reabsorbed along the early midcolon (Fig. 5D). The localized enrichment of Cl^- in the proximal segment cannot be ascribed

to either retrograde or upstream inflow, since the stool in both flanking segments contains much less Cl^- . The most likely sources of this added Cl^- are the crypt cells of the proximal colon. A local recirculation of Cl^- , along with osmotically entrained water, from the crypt to the surface could provide for optimal stool liquidity or luminal flushing. It could also furnish extra Cl^- to sustain the coupled absorption of luminal solutes (e.g., via paired Na/H and Cl/HCO_3 exchanges) by surface epithelial cells downstream (i.e., in the midcolon).

Midcolon (20–60% colon length). Our studies indicate that NHE3 and DRA coexist at high levels only in the middle third of the rodent colon (Figs. 2 and 3). Thus the conventional model for electroneutral NaCl absorption in the colon, which postulates functional coupling between NHE3 and DRA, appears to be applicable only to this particular segment of the rodent colon. The focal pairing of NHE3 and DRA activities in the rodent midcolon is consistent with evidence that the stool surrenders considerable NaCl and water as it traverses this region in a manner that preserves a near neutral pH_{ms} (Fig. 5). The cooperative operation of NHE3 and DRA (62) could reflect the known ability of these proteins to form a molecular complex via the adaptor protein E3KARP (53). Intimate proximity would also allow for efficient regulatory coupling, as each component would be allosterically stimulated, via changes in regional intracellular pH, by the transport activity of the other; thus the intracellular acidifying effect of Cl/HCO_3 exchange would tend to stimulate NHE3 (84) and the alkalinizing effect of NHE3 would tend to stimulate DRA (17, 37). Like other segments, the rodent midcolon exhibits high CA activity, with contributions from various CA isoforms situated in the cytosol, the inner and outer surfaces of the apical membrane, and the luminal mucus layer (49). The anion exchange activity of DRA is known to be enhanced by cytosolic CA-II, presumably by furnishing H^+ and HCO_3^- for exchange with Na^+ and Cl^- , although this does not appear to involve a direct interaction (78).

Distal colon (60–100% colon length). Our finding that the rodent distal colon, like the cecum, expresses high levels of DRA but negligible NHE3 is compatible with previous reports that this segment, like the cecum, can rapidly secrete HCO_3^- in a manner that is electroneutral, coupled to Cl^- absorption (7, 27, 28, 46, 66, 83), and dependent on DRA function (74, 79). Our results, however, are inconsistent with previous evidence that NHE3, but not NHE2, participates in electroneutral HCO_3^- -dependent NaCl absorption by the rat distal colon (5, 51); the experimental use of proximal regions of the distal colon, in which NHE3 is low but clearly present (Fig. 2), could account for this discrepant finding.

Thermodynamic considerations. The extent to which DRA actually contributes to Cl^- absorption and HCO_3^- secretion in the cecum and distal colon in vivo remains uncertain. First, the passage of stool from terminal ileum to cecum to proximal colon, and from midcolon to distal colon, is not associated with significant losses of Cl^- ions or water (Fig. 5). Second, the quantity of Cl^- within cecal stool that would be available for exchange with HCO_3^- is rather limited (<20% that of SCFAs). Third, the capacity of DRA for forward operation, i.e., removal of luminal Cl^- and addition of luminal HCO_3^- , may become energetically limited in the cecum and distal colon, where luminal $[\text{Cl}^-]$ is low. A precise assessment of the thermodynamic forces on Cl/HCO_3 exchange is not yet possible owing

to uncertainties of luminal $[\text{HCO}_3^-]$, intracellular $[\text{Cl}^-]$ and $[\text{HCO}_3^-]$, and DRA coupling stoichiometry (76) along the colonic axis.

Our findings contrast with some earlier reports that NHE3 and DRA are expressed throughout the entire colon. It is apparent from Fig. 2 that this discrepancy could result from the common experimental practice of harvesting equal proximal and distal halves of the colon and of omitting the extraperitoneal segment that extends from the pelvic brim to the anus. In these cases, both halves would include similar portions of the midcolon that expresses both exchangers. It would seem preferable to distinguish colonic segments on the basis of their distinctive functional properties, as was proposed decades ago (30), or on their distinctive patterns of transporter expression, as conceptualized in Fig. 6. The functional segments demarcated by our study appear to have two easily identifiable anatomical features. First, the proximal portion of the colon that expresses abundant NHE3 but scant DRA (0–20% length) roughly corresponds to the segment that displays serosal palm-leaf striations (21, 31, 55). Second, the portion of the rodent distal colon that is deficient in NHE3 (60–100% colon length in rats and 75–100% in mice) is largely comprised of the extraperitoneal segment that extends from the pelvic brim to the anus; previous studies have indicated that this segment, sometimes called the rectum (30), uniquely exhibits aldosterone-stimulated electrogenic Na^+ absorption and a weaker Cl^- secretory capacity (7, 30, 43).

In summary, the reciprocal profiles of NHE3 and DRA expression along the colonic axis and the segmental variations in mucosal surface pH and $[\text{Na}^+]$ suggest a hypothetical model (Fig. 6) in which 1) cecal HCO_3^- secretion via unpaired DRA neutralizes excess fermentative H^+ ; 2) uncoupled NHE3 activity in the early proximal colon preserves an acidic microenvironment that energizes SCFA $^-$ absorption via apical SCFA/ HCO_3 exchange and nonionic diffusion of protonated SCFA; and 3) coupled NHE3/DRA activities in the middle third of the colon provide for rapid NaCl absorption. Our results also suggest that crypt cells within the early proximal colon add substantial amounts of Cl^- (and possibly water) to the stool in vivo, presumably through crypt-based Cl^- secretion; this could provide for optimal luminal liquidity or furnish extra Cl^- to sustain downstream Cl/HCO_3 exchange.

The organization of the distal gut into functionally distinct compartments is expected to figure prominently in the well-being of the colon and its microbial inhabitants. Regional epithelial transport could influence the chemical composition and physical properties of the luminal contents, especially within the juxtamucosal microenvironment where convective mixing is minimum, and thereby shape the growth and stratification of the microbial communities along the gut (26, 59).

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DISCLOSURES

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