slc26a3 (dra)−deficient Mice Display Chloride-losing Diarrhea, Enhanced Colonic Proliferation, and Distinct Up-regulation of Ion Transporters in the Colon*

Mutations in the SLC26A3 (DRA (down-regulated in adenoma)) gene constitute the molecular etiology of congenital chloride-losing diarrhea in humans. To ascertain its role in intestinal physiology, gene targeting was used to prepare mice lacking slc26a3. slc26a3-deficient animals displayed postpartum lethality at low penetrance. Surviving dra−/−deficient mice exhibited high chloride content diarrhea, volume depletion, and growth retardation. In addition, the large intestinal loops were distended, with colonic mucosa exhibiting an aberrant growth pattern and the colonic crypt proliferative zone being greatly expanded in slc26a3-null mice. Apical membrane chloride/base exchange activity was sharply reduced, and luminal content was more acidic in slc26a3-null mouse colon. The epithelial cells in the colon displayed unique adaptive regulation of ion transporters; NHE3 expression was enhanced in the proximal and distal colon, whereas colonic H,K-ATPase and the epithelial sodium channel showed massive up-regulation in the distal colon. Plasma aldosterone was increased in slc26a3-null mice. We conclude that slc26a3 is the major apical chloride/base exchanger and is essential for the absorption of chloride in the colon. In addition, slc26a3 regulates colonic crypt proliferation. Deletion of slc26a3 results in chloride-rich diarrhea and is associated with compensatory adaptive up-regulation of ion-absorbing transporters.

The SLC26A3 or DRA (down-regulated in adenoma) gene was originally identified in a subtractive hybridization screen comparing the mRNAs expressed in colon cancer and normal colon tissue (1). DRA is expressed in normal colonic epithelium, but is absent or reduced in adenomas and adencarcinomas (1). Subsequent studies identified SLC26A3 (DRA) as a member of a large conserved family of anion exchangers (SLC26) that encompass at least 10 distinct genes (2–20). Except for SLC26A5 (prestin), all function as anion exchangers with versatility with respect to transported anions (2–20). Immunohistochemical studies localized SLC26A3 on the apical membrane of colonic mucosa, with lower levels in the small intestine (4, 25). In humans, SLC26A3 encodes a 764-amino acid protein and is located on chromosome 7 in a head-to-tail arrangement with SLC26A4 (pendrin), indicating ancient gene duplication.

Genetic analysis studies linked mutations in DRA (SLC26A3) to congenital chloride-losing diarrhea (CLD; OMIM accession number 214700), a disease manifested by enhanced chloride loss in the stool and volume depletion (4). Functional studies in vitro have demonstrated that SLC26A3 can mediate multiple anion exchange modes, including Cl−/HCO3−, Cl−/oxalate, and Cl−/hydroxyl, and possibly sulfate/hydroxyl exchanges (6, 21–26). Similar anion exchange activities have been described previously in apical membranes of the colon (27, 28), the site of abundant DRA expression.

To initiate an investigation into the role of DRA in an in vivo model, we created slc26a3 (dra)−/−gene-targeted mice that are null for expression of the slc26a3 gene. This mouse model closely resembles the clinicopathological presentation of human CLD. Functional studies demonstrated that slc26a3−/−null mice have significantly reduced levels of apical chloride/base exchange activity in the colon and display unique and distinct adaptive regulation of ion transporters in the proximal and distal colon. In addition, the loss of slc26a3 produces an expansion of the proliferative zone of the colonic crypt epithelium, suggesting a role for loss of slc26a3 expression in colon tumor progression.
CLD and Ion Transporter Regulation in dra-null Mouse Colon

FIGURE 1. Targeted disruption of the mouse slc26a3 gene. A, Southern blot analysis of 10 μg of genomic DNA from ES cells stably transfected with the targeting vector containing the neo cassette. Two lanes representing ES cell clones containing a targeted disruption of one dra allele are marked with asterisks. A schematic representation of the targeting vector showing the neo cassette inserted into Dra exon 2 and the flanking probe used for hybridization is shown below the Southern blot. Shown are the neo cassette introduced and the EcoRV site that resulted in a 10.5-kbp EcoRV fragment in the targeted allele versus an 18.5-kbp fragment in the normal allele. B, genotype analysis by PCR of tail DNA from mice derived by a heterozygous mating (dra+/− × dra+/−). The PCR products shown on a 45 mM Tris borate and 1 mM EDTA (pH 8.0)-containing 3% agarose gel contained both alleles (220 and 115 bp) in heterozygous (ht) mice, only the 115-bp allele in wild-type (wt) mice, or only the 220-bp allele in homozygous (hm) KO mice. A negative control lane (−ve) with no template DNA is also shown. The molecular weight markers were derived from an HaeIII digest of DX174 DNA and represent 310 bp, a 281/271-bp doublet, 234 bp, 194 bp, 118 bp, 72 bp, and 18 bp. A schematic representation of the PCR strategy in Dra exon 2 (with or without the neo cassette) is also shown. C, Northern blot analysis of 5 μg of total RNA from wild-type and homozygous mouse ileum (i) and colon (c) tissues. The blot was probed with mouse Dra cDNA. Two exposures are shown: 6 h (upper panel) and 2 days (lower panel).

TABLE 1

Distribution of genotypes among 409 progeny of dra heterozygous matings

| Genotype | No. of offspring | χ² | p value |
|----------|-----------------|----|--------|
|          | Observed        |     |        |
| dra+/−   | 166             | 102.25 |       |
| dra+/−   | 179             | 204.50 |       |
| Total    | 409             | 409 | 57.235 | <0.0001* |

* χ² was calculated as ∑(observed − expected)²/expected.

* The p value was determined from an unpaired Student’s t test (two-tailed).

EXPERIMENTAL PROCEDURES

Isolation of Mouse slc26a3 Genomic Clones—A mouse 129S6/SvEv strain genomic library in the vector Lambda Dash II (Stratagene, La Jolla, CA) was screened by plaque hybridization using human DRA cDNA as a probe. A 19-kbp fragment containing at least exons 2–9 was isolated. The identity of the clones was confirmed by Southern blot hybridization and partial sequencing.

Targeting Vector Construction—Restriction enzyme mapping revealed the presence of an Agel restriction site on exon 2 of the mouse dra genomic DNA that was suitable for insertion of the neo cassette. The neo cassette contains the neomycin phosphotransferase gene under the transcriptional control of the RNA polymerase II promoter and the last exon and polyadenylation signal of the hypoxanthine-guanine phosphoribosyltransferase gene for termination. In addition, the neo cassette is flanked by the loxP/Cre recombinase recognition sequences (kindly provided by K. Thomas). The Agel site of the neo cassette insertion is located 39 bp downstream of the dra gene. The targeting vector contains 5.6 kbp of isogenic genomic DNA upstream and 8 kbp of isogenic genomic DNA downstream of the Agel site. The targeting vector was linearized with SfiI, which cleaves the vector backbone, prior to electroporation into embryonic stem (ES) cells.

ES Cell Electroporation—TC1-10 ES cells (kindly provided by Dr. P. Leder) were grown on feeder layers of mouse fibroblasts in knockout Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 15% serum replacement medium and made complete as described previously (29). ES cells (2 × 10⁷) were electroporated with 68 μg of linearized vector using a Bio-Rad Gene Pulser at 600 V and 25 microfarads. Transfected cells were treated with G418 (230 μg/ml) for positive selection. Drug-resistant ES cell clones were expanded and screened for homologous recombination by Southern blot analysis of their genomic DNA (see Fig. 1A). Three of 40 drug-resistant clones were generated and identified by this method.

Generation of slc26a3 (dra) Knock-out Mice—The ES cells from three of the positive colonies were microinjected into C57BL/6j blastocysts and implanted in the uteri of pseudo-pregnant female mice. Chimeric males that demonstrated significant agouti coat color were mated with BL6 females to generate heterozygotes, which were initially identified by the same Southern blot strategy used to identify targeted ES cell lines. Subsequently, the slc26a3 genotyping was conducted by PCR using two slc26a3 exon 2-specific primers and a primer specific for the RNA polymerase II gene (see below).

Genotyping of Mice—Genotyping was performed on tail DNA obtained from mice at weaning and placed directly into 250 μl of lysis buffer (10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.45% Nonidet P-40, and 0.45% Tween 20). PCR was performed with the following primers: mouse slc26a3 exon 2, 5′-GGCAAAATGATCGAAGCCAT-3′ (forward) and 5′-GATGGTCAGGAAATGCTTGGATGTC-3′ (reverse); and neo cassette RNA polymerase II,
5′-GGAAAGTAGCGTATTTAGTCGAGG-3′ (reverse). The PCR products were as follows: 95 °C for 3 min, followed by 32 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The PCR products were electrophoresed on 3% low melting point agarose (see Fig. 1B) in 45 mM Tris borate and 1 mM EDTA (pH 8.0).

**Luminal Chloride, Sodium, and pH Measurement**—The chloride assay was performed using a diagnostic kit obtained from Sigma (catalog no. 461-3) according to the manufacturer’s instructions. Briefly, stool samples from the animals were collected, weighed, lyophilized, weighed again, resuspended in water at 5–100 mg/ml, and then heated to 65 °C for 30 min. The samples were centrifuged in a tabletop centriuge, and the supernatant was removed and assayed for chloride. The sodium assay was performed by flame photometry. The pH of the intestinal content was assayed using a pH microelectrode.

**Histopathological Examination of Mouse Tissues**—Mice were killed, and tissues were harvested within 10 min. Tissues were fixed in Amsterdam solution and then processed and embedded in paraffin blocks using standard methods. Sections were cut and stained using hematoxylin/eosin or subjected to immunohistochemical/immunofluorescence labeling as described (13, 20). The primary antibodies used were anti-SLC26A3, anti-NHE3, and anti-SLC26A6.

**RNA Isolation and Northern Blot Hybridization**—Total cellular RNA was extracted from various mouse tissues, including ileum, proximal and distal colon, duodenum, and kidney, according to established methods; quantitated spectrophotometrically; and stored at −80 °C. Total RNA samples (5–30 µg/lane) were fractionated on a 1.2% agarose gel containing formaldehyde, transferred to Magna NT nylon membranes, cross-linked by UV light, and baked. Hybridization was performed according to established protocols (30). The membranes were washed, blotted dry, and exposed to a PhosphoImager screen (GE Healthcare). The following DNA fragments were used as specific probes for Northern hybridization: for NHE3, a fragment encoding nucleotides 1883–2217; for SLC26A6, a fragment encoding nucleotides 51–488; for colonic H,K-ATPase, three pooled PCR products from rat (nucleotides 135–515, 2369–2998, and 3098–3678); and for ENaCα, ENaCβ, and ENaCγ, fragments encoding nucleotides 1197–1890, 58–671, and 135–790, respectively. Each Northern hybridization was performed on separate samples from three different animals.

**Immunoblotting and/or Immunohistochemical Labeling of slc26a3 and nhe3 in Mouse Intestine**—slc26a3+/– and slc26a3–/– mice were killed with a sodium pentobarbital overdose and perfused through the left ventricle with 0.9% saline, followed by cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Intestinal segments were removed, cut into tissue blocks, and fixed overnight in formaldehyde solution at 4 °C. The tissues were frozen on dry ice, and 6-µm sections were cut with a cryostat and stored at −80 °C until used. Immunohistochemical labeling was performed as described (13) using SLC26A3-specific antibodies. For immunoblot analysis, microsomal membranes from the proximal colon were resolved by SDS-PAGE, and membranes were blotted with anti-SLC26A3 or anti-NHE3 antibodies. Each blot was performed on three separate samples from three different animals.

**36Cl and 22Na Transport Measurement**—The uptake of 36Cl or 22Na by luminal membrane vesicle suspensions, pooled from the proximal colons of three mice and prepared according to established methods (17, 27, 28), was assayed at room temperature in triplicate by the rapid filtration technique (32). The reaction was stopped by ice-cold medium. The radioactivity in each filter was assayed by scintillation spectroscopy. Vesicles and all experimental media were continuously gassed with 100% N2 or 5% CO2 and 95% N2. The uptake of 4 mM 36Cl was measured under three different conditions: no pH gradient (pHi/Pho 7.5/7.5 without CO2/HCO3−), outward pH gradient (pHi 7.5/Pho 6.0 without CO2/HCO3−), and outward pH and bicarbonate gradients (pHi 7.5/Pho 6.0 with CO2/HCO3−). The bicarbonate concentrations were 25 mM at pH 7.5 and 0 mM at pH 6.0. The uptake of 1 mM 22Na was measured under two different conditions: no pH gradient (pHi 7.5/Pho 7.5) and inward pH gradient (pHi 6.0/Pho 7.5) without CO2/HCO3−. Each radioactive uptake experiment was repeated on three different samples.

**Materials**—[32P]dCTP, 36Cl, and 22Na were purchased from Perkin-
Elmer Life Sciences. Nitrocellulose filters and other chemicals were purchased from Sigma. The RadPrime DNA labeling kit was purchased from Invitrogen. Anti-SLC26A6 antibodies was raised in our laboratories (13). Antibodies against SLC26A3 peptides were custom-generated by Zymed Laboratories Inc. (South San Francisco, CA) as described (33). Anti-NHE3 polyclonal antibodies were purchased from Chemicon.

**Statistical Analyses**—Values are expressed as the means ± S.E. Statistical analysis was examined using Student’s t test or analysis of variance. p < 0.05 was considered statistically significant.

**RESULTS**

An slc26a3 gene-targeted allele was generated in ES cells using a targeting vector containing the neo cassette inserted into exon 2 of an isogenic 129 strain dra genomic clone as shown in Fig. 1A. Three germ line chimeric founder males were generated from dra gene-targeted ES cells and were used to breed in a C57BL6 background. Genotype analysis of offspring was performed by a three-primer PCR using the strategy shown in Fig. 1B. The targeted allele produced a 220-bp PCR fragment, whereas the wild-type allele produced a 115-bp fragment. Fig. 1B shows a typical genotyping demonstrating wild-type, heterozygous, and homozygous knockout (KO) animals.

DRA is expressed in a limited subset of tissues, including small and large intestines, intraprostatic seminal vesicles, and eccrine sweat glands (4–6, 33, 34). However, the intestines express DRA to the greatest degree (4–6, 33). Therefore, to test for generation of a null allele via gene targeting, we performed Northern blot analysis on RNAs from the ilea and colons of wild-type and homozygous animals (Fig. 1C). The results showed that the homozygous individuals were completely devoid of DRA mRNA expression. Heterozygous intercross mating was performed to determine whether the dra KO allele is transmitted to the subsequent generation in Mendelian ratios. Table 1 shows the results of genotype analysis of 409 such offspring at weaning and reveals a non-Mendelian distribution of genotypes (40.6% wild-type, 43.8% heterozygous, and 15.6% KO). The deviation from Mendelian ratios is highly significant (p < 0.0001) for both heterozygous and homozygous animals, suggesting a dra gene dosage affect on viability, albeit at reduced penetrance.

Table 2 shows the results of mating between heterozygous and homozygous KO littermates. Again, the weaned offspring demonstrated a non-Mendelian distribution that is a highly significant departure from expected Mendelian ratios (p = 0.0002).

To determine whether dra disruption affects the gross morphology of the developing embryo, we examined the intestinal morphol-

![Fig. 3I (panels A-D).](image1)

![Fig. 3II (panels A-E).](image2)

**FIGURE 3.** Morphological analysis of colons from slc26a3−/− mice. I, colon dilatation shows the distended proximal (panels A and C) and distal (panel B) colons of dra−/− mice and the proximal colon in a normal mouse (panel D) immediately after death and dissection. Arrows point to the colon in each panel. Scale bar = 0.5 cm. II, whole mount preparation of the colon mucosal surface. Panels A–C and panel D show the mucosal surface of dra−/− and normal mice, respectively, stained with methylene blue. Panels A and B are shown at ×40 magnification. Scale bar = 500 μm. Panels C and D are shown at ×100 magnification. Scale bars = 100 μm. Panel E demonstrates serial sectioning (8 μm) through conjoined crypts. Panel E is shown at ×100 magnification. Scale bars = 100 μm.
ogy during embryogenesis. We have shown previously that dra gene expression commences in the gut at embryonic day 16.5 postcoitus (33). Therefore, we harvested embryos at day 18.5 postcoitus. There were no detectable differences in the gross morphology or architecture of the intestines of dra-deficient mice and their normal siblings (data not shown). The expected Mendelian ratios for heterozygous mating was observed among the embryos (data not shown), suggesting that postpartum lethality is responsible for the non-Mendelian ratios observed later.

Surviving dra-deficient mice exhibited diarrhea with elevated chloride content. Fig. 2A shows that the chloride level of stool specimens from dra-deficient mice (mean ± S.D. of 28.37 ± 6.66 μg/mg (dry weight)) was significantly higher (p < 0.0001) than that from wild-type and heterozygous animals (mean ± S.D. of 1.87 ± 0.79 μg/mg (dry weight)). In addition, stools from the dra-deficient mice had a higher water content compared with those from their control littermates (p < 0.0001) (Fig. 2B), although the stools were not as watery as described in human CLD. In addition, homozygous animals occasionally developed prolapsed rectums. Finally, dra-deficient mice were smaller compared with age-matched heterozygous and wild-type animals (p = 0.0007) (Fig. 2C) and had greatly shortened life spans (mean ± S.D. of 121.6 ± 50.8 days). These observations are consistent with the conditions in human CLD, which is characterized by voluminous watery diarrhea with high chloride content and growth retardation in untreated individuals (35). Patients treated with electrolyte replacement continue to have watery, high chloride content diarrhea, but have normal life...
spans (35). Mutations in human DRA are the cause of CLD (4, 5, 34). Therefore, the dra-deficient mouse model closely resembles the molecular etiology and clinical manifestations of human CLD.

Other morphological and histological aspects distinguished the dra KO mice from normal animals. Upon immediate post-death dissection, dra-deficient mice presented with distension of the large bowel (Fig. 3I, panels A–C) compared with wild-type mice (panel D). The small intestine was not affected in this manner. Such intestinal dilation is consistent with pre- and postpartum observations of human CLD and is due to increased volume of the bowel contents (35). We next examined the morphology of the colonic tissue. Whole mount preparations of colons that were sliced open longitudinally, fixed, and stained with methylene blue revealed a starkly altered mucosal morphology in dra-deficient mice compared with those in heterozygous and wild-type mice. Fig. 3II (panels A–C) shows an abnormal growth pattern of the mucosal surface wherein the crypt orifices were conjoined rather than distinct as in normal mucosa (panel D). This conjoined phenotype was observed in sections from both the proximal and distal colons of KO mice, but not in the ilea (data not shown). Fig. 3II (panel E) demonstrates serial sections (8 μm) down through an example of conjoined crypts. It shows that the conjoined crypt phenotype occurred at the very top of the crypt, which is closest to the lumen (panel E).

SLC26A3 functions predominantly as a chloride/base exchanger in vitro (6, 21–23). To ascertain the role of SLC26A3 in mediating chloride/base exchange in vivo, apical membrane vesicles were isolated from the proximal colons of wild-type and slc26a3-null mice and assayed for chloride/base exchange using the 36Cl influx method. As demonstrated in Fig. 4A, the...
influx of radiolabeled chloride mediated via Cl\(^–/\)OH\(^–\) and Cl\(^–/\)HCO\(_3\) exchange decreased by 76 and 69%, respectively, in apical membrane vesicles from the colons of slc26a3-null mice (\(p < 0.001\) versus wild-type mice). The reduction in apical chloride influx correlated with results from immunoblot analysis demonstrating the complete absence of slc26a3 protein in the colons of KO mice (Fig. 4E).

Despite a very watery cecal content, the final stools were less watery, suggesting an increased absorptive capacity of the colons in slc26a3 KO mice. To ascertain the molecular mechanism of enhanced electrolyte absorption in the colon, the expression and/or activities of the apical Na\(^+\)/H\(^+\) exchanger NHE3, the electrogenic sodium channel, and colonic H,K-ATPase were examined. NHE3 mRNA expression was increased by 2- and 3-fold in the proximal and distal colons, respectively, from slc26a3-null mice (\(p < 0.001\) versus wild-type mice; \(n = 4\)) (Fig. 4C). Immunoblotting confirmed the up-regulation of NHE3 in the proximal colons of slc26a3 KO mice (Fig. 4D). The enhanced expression of NHE3 was associated with increased Na\(^+\)/H\(^+\) exchange activity in luminal membrane vesicles isolated from the colons of slc26a3 KO mice (Fig. 4E).

Colonic H,K-ATPase is expressed in the distal colon and is predominantly responsible for the absorption of potassium. Colonic H,K-ATPase mRNA was increased by \(-4\)-fold in the distal colons from slc26a3 KO mice (\(p < 0.001\) versus wild-type mice; \(n = 4\)) (Fig. 4F). In addition to NHE3 and colonic H,K-ATPase, an electrogenic Na\(^+\) channel is expressed in the apical membranes of the distal colon and is involved in the absorption of sodium. Northern analysis showed significant up-regulation of the ENaC \(\alpha\), \(\beta\), and \(\gamma\)-subunits in the distal colons of slc26a3 KO mice (\(p < 0.001\) versus wild-type mice; \(n = 4\)) (Fig. 4G).

Histological and immunohistochemical analyses of the colonic crypts further distinguished slc26a3-deficient animals from their normal littermates. By these methods, we determined the expression patterns for dra and the retinoblastoma protein and p53 tumor suppressor genes, the proliferating cell nuclear antigen proliferation marker, and incorporated bromodeoxyuridine. In addition, the expression of cyclins A, B\(_1\), D\(_1\), E, and H, and cyclin-dependent kinase inhibitory proteins p27, p21, and p16 was examined. Most of these proteins showed similar expression patterns in all three dra genotypes (data not shown). However, as shown in Fig. 5, several had notable differences that distinguished the homozygous mice from the heterozygous and wild-type mice. In wild-type normal mice, slc26a3 expression was localized to the apical membrane of the upper crypt columnar epithelium (Fig. 5). slc26a3 expression was completely absent in KO mice (Fig. 5). Occasionally, dra expression was detected in a few cells descending into the crypt (data not shown). This localization is similar to that observed for human DRA in normal colon tissue (33, 36). The proliferative zone in the lower crypt was expanded in dra KO mice (Fig. 5). Both proliferating cell nuclear antigen staining and bromodeoxyuridine incorporation indicated that the expanded zone occupied one-third to one-half of the crypt axis. In contrast, normal colon tissue contained only a small number of proliferating stem cells that were confined to the bottom one-quarter of the crypt. This result was unexpected because dra was not expressed in the lower crypt (which includes the proliferative zone) in normal colon tissue. Concomitant with the observed expansion of the proliferative zone, cyclin A was also expressed in many more cells in the lower one-third of the crypt compared with minimal expression in normal colon tissue (Fig. 5). Together, these results demonstrate that loss of DRA expression alters the overall proliferative homeostasis of the colonic crypt epithelium.

Table 3 summarizes the serum electrolyte profile and parameters of kidney function (including blood urea nitrogen and serum electrolytes, blood urea nitrogen, and aldosterone levels

| Genotype | Na\(^+\) | Cl\(^–\) | K\(^+\) | BUN | Serum aldosterone |
|----------|---------|---------|-------|-----|------------------|
| DRA\(^{+/+}\) | 153 ± 1.5 | 115 ± 1.1 | 5.6 ± 0.4 | 16 ± 1 | 0.45 ± 0.07 |
| DRA\(^{++}\) | 142 ± 1.3\(a\) | 102 ± 1.4\(a\) | 5.1 ± 0.4\(a\) | 37 ± 3\(a\) | 2.3 ± 0.2\(a\) |

\(a\)p < 0.05.
\(b\)p < 0.01.
clade) were determined for four different animals. slc26a3-null animals had severe volume depletion with consequent renal failure as determined by increased serum concentrations of blood urea nitrogen and aldosterone. Serum sodium and chloride concentrations were significantly decreased in slc26a3 KO mice (Table 3).

**DISCUSSION**

The DRA gene appears to possess two independent functions: one as an anion transporter that principally mediates Cl⁻/HCO₃⁻ exchange in the large intestine (6, 21–23) and the other as a regulator of proliferation of epithelial colonocytes by an unknown mechanism (37). Our dra KO mouse model closely resembles the human disease CLD in several important clinicopathological aspects, including stool chloride concentration, diarrhea, and growth retardation.

Consequent to diarrhea, humans with CLD become dehydrated as manifested by decreased kidney perfusion and hypoelectrolytemia. slc26a3 KO mice displayed evidence of severe dehydration as determined by increased serum aldosterone levels (Table 3) and increased expression of renin in the kidney (data not shown). Furthermore, urine osmolarity increased in slc26a3-null animals (data not shown), consistent with increased vasopressin levels, a known response to dehydration. Finally, serum sodium and chloride concentrations were decreased in slc26a3 KO mice because of their loss in the colon. Taken together, these results clearly support the notion that deletion of dra recapitulates the human CLD phenotype in mice.

We observed that the surface mucosa in dra-deficient mice had an altered pattern suggestive of hyperplasia. This pattern was limited strictly to the surface mucosa because sections along the longitudinal crypt axis had a normal architecture (Fig. 5). Also, there was no evidence of renewed proliferation in the columnar epithelial cells that arise via differentiation and withdrawal from the cell cycle. The surface mucosa did not show evidence of aberrant crypt foci as seen in azoxymethane-induced colon carcinogenesis (38–40) or the variant aberrant crypt foci observed in the ApcMin mouse model of intestinal cancer (41–43). Thus, the phenotype observed in Dra-deficient mice is novel. Normally, the lifespan of mouse colonic enterocytes is ~3–4 days before the cells undergo apoptosis and slough off into the intestinal lumen (44). Therefore, one possibility is that the surface epithelium in Dra⁻/⁻ mice has reduced apoptotic activity. Alternatively, the expanded proliferative zone (see below) may generate more epithelial cells than normally removed by apoptosis, thereby altering the equilibrium of the total number of mucosal cells. As such, an “excess” of cells migrating to the surface epithelium may produce the conjoined phenotype. This latter alternative is consistent with the data presented.

The proliferative zone in the colonic epithelium of Dra-deficient mice was expanded (Fig. 5). This was confirmed using two independent measures of proliferation (proliferating cell nuclear antigen staining and bromodeoxyuridine incorporation) (Fig. 5). The unexpected aspect of this observation is not that loss of Dra resulted in increased proliferation in the mucosal epithelium; we reported recently that human colon cancer cells transfected with human DRA cDNA are growth-suppressed (37). Rather, it is that such proliferation was observed in the lower half of the crypt instead of the upper crypt and the columnar epithelium, where dra is more abundant. In our dra KO model, the cells that normally express dra are already terminally differentiated (Fig. 3), thus they may be less likely to re-enter the cell cycle without additional genetic changes. The observation that this expanded proliferative zone does not normally express DRA (33) or may express low levels of DRA (4, 25) may point to the multigenetic basis for neoplastic progression. It is plausible that alteration of the intracellular and/or extracellular milieu resulting from enhanced acid secretion via activation of the Na⁺/H⁺ exchanger and H,K-ATPase and/or decreased chloride/bicarbonate exchange activity due to DRA deletion might enhance DNA synthesis and cell proliferation. In support of such a possibility, generation of intracellular alkalinization via activation of the Na⁺/H⁺ exchanger has been shown to enhance DNA synthesis in colonocytes and hepatocytes (45–47).

Apical Cl⁻/OH⁻ and Cl⁻/HCO₃⁻ exchange activities were significantly decreased in the colons of slc26a3-null mice (Fig. 3), resulting in increased fecal chloride, sodium, and water loss. Comparison of the luminal content in the cecum with that in the final stool indicated increased absorption of fluid and electrolytes in slc26a3-null mouse colon, consistent with compensatory up-regulation of electrolyte-absorbing transporters. Three major sodium- and/or potassium-absorbing transporters in the colon are the Na⁺/H⁺ exchanger NHE3, ENaC, and colonic H,K-ATPase, which showed impressive up-regulation in slc26a3 KO mice. The reduction in the apical chloride/base exchange activity and up-regulation of NHE3 and colonic H,K-ATPase could explain the acidic colonic luminal content in dra KO mice (pH 6.2 ± 0.15 in KO mice and pH 7.25 ± 0.20 in wild-type mice (n = 4); p < 0.02).

The schematic diagram in Fig. 6 depicts these adaptive processes. As noted, the coordinated up-regulation of colonic H,K-ATPase and ENaC can result in enhanced absorption of sodium in the distal colon, with potassium recycling providing the driv-
ing force for continued sodium absorption via ENaC. This is consistent with the published role of colonic H,K-ATPase in the electrogenic absorption of sodium (48). The up-regulation of NHE3 in the proximal and distal colon may be an attempt to minimize the loss of sodium resulting from chloride loss. Taken together, these data show that SLC26A3 is the major absorptive chloride/base exchanger in the colon and that its absence impairs Na\(^+\)-fluid volume homeostasis.

Although the colonic apical chloride/base exchange activity was sharply reduced in slc26a3 KO mice, significant residual activity was detected in membrane vesicles isolated from this segment (Fig. 4), raising the possibility that other apical anion exchangers may be up-regulated. One obvious candidate is the small intestinal apical Cl\(^-\)/HCO\(_3^-\) exchanger SLC26A6 (PAT1), which is exclusively expressed in the duodenum, jejunum, and ileum, but is absent in the large intestine (13, 17). We did not detect any expression of SLC26A6 in the colons of slc26a3 KO mice (data not shown). Intriguingly, a 3-fold up-regulation of PAT1 expression was detected in the small intestines of dra-null mouse colon (Fig. 4), raising the possibility that other apical anion exchangers may play an important compensatory role in electrolyte homeostasis. Other SLC26 isoforms such as SLC26A4, SLC26A8, and SLC26A11 did not show any compensatory up-regulation in the colons ofdra KO mice.

In conclusion, our slc26a3 KO mouse model closely resembles human CLD and produces chloride-rich diarrhea, dehydration, and distinct adaptive regulation of ion transporters in the colon. In addition, deletion of slc26a3 causes alterations in the proliferation of the colonic crypt epithelium in a manner that suggests a role for DRA in colonic crypt function and homeostasis.

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