Ileal oxalate absorption and urinary oxalate excretion are enhanced in Slc26a6 null mice

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Freel, Robert W., Marguerite Hatch, Mike Green, and Manoocher Soleimani. Ileal oxalate absorption and urinary oxalate excretion are enhanced in Slc26a6 null mice. Am J Physiol Gastrointest Liver Physiol 290: G719–G728, 2006; doi:10.1152/ajpgi.00481.2005.—Intestinal oxalate transport, mediated by anion exchange proteins, is important to oxalate homeostasis and consequently to calcium oxalate stone diseases. To assess the contribution of the putative anion transporter (PAT1) (Slc26a6) to transepithelial oxalate transport, we compared the unidirectional and net fluxes of oxalate across isolated, short-circuited segments of the distal ileum of wild-type (WT) mice and Slc26a6 null mice [knockout (KO)]. Additionally, urinary oxalate excretion was measured in both groups. In WT mouse ileum, there was a small net secretion of oxalate (Jox net = −5.0 ± 5.0 pmol·cm⁻²·h⁻¹), whereas in KO mice Jox net was significantly absorbptive (75 ± 10 pmol·cm⁻²·h⁻¹), which was the result of a smaller serosal-to-mucosal oxalate flux (Jsm ox) and a larger mucosal-to-serosal oxalate flux (Jms ox). Mucosal DIDS (200 μM) reduced Jms ox in WT mice, leading to reversal of the direction of net oxalate transport from secretion to absorption (Jsm ox = 15.0 ± 5.0 pmol·cm⁻²·h⁻¹), but DIDS had no significant effect on KO ileum. In WT mice in the absence of mucosal Cl⁻, there were small increases in Jms ox and decreases in Jsm ox that led to a small net oxalate absorption. In KO mice, Jsm ox was 1.5-fold greater in the absence of mucosal Cl⁻, due solely to an increase in Jsm ox. Urinary oxalate excretion was about fourfold greater in KO mice compared with WT littermates. We conclude that PAT1 is DIDS sensitive and mediates a significant fraction of oxalate efflux across the apical membrane in exchange for Cl⁻; as such, PAT1 represents a major apical membrane pathway mediating Jsm ox.

putative anion transporter 1; hyperoxaluria; anion exchange; serum oxalate; 4,4′-disothiocyanostilbene-2,2′-disulfonic acid

THE MAMMALIAN INTESTINE plays a significant role in the homeostasis of the oxalate anion both as a site for dietary oxalate absorption and, together with renal mechanisms, as an avenue for the excretion of oxalate (11). Understanding the mechanisms and regulation of intestinal oxalate transport is thus an important component in the management of hyperoxaluria and calcium oxalate urolithiasis (11). From studies of isolated, short-circuited intestinal epithelia from rats and rabbits, it is clear that transepithelial oxalate transport occurs passively through paracellular pathways and actively (secondarily) through tranacellular pathways in a vectorial manner that produces net absorption or net secretion of oxalate in a segment-specific fashion (6, 12, 13, 15). The latter studies, and others employing membrane vesicles prepared from rabbit distal ileum (19, 20), suggested that tranmembrane oxalate transport across the apical and basolateral membrane is mediated by one or more distinct anion exchange systems (antiporters) that exhibit varying degrees of stilbene sensitivity. The apparent multiplicity of exchangers at a given membrane, the variety of possible cotransport partners (exchange modes), and the general inability to identify a single specific exchanger (beyond stilbene sensitivity or exchange modes) have hampered a completely satisfactory explanation of net oxalate absorption or secretion by intestinal or renal epithelia.

The molecular identification of the individual pathways involved in transepithelial oxalate transport has become more promising with the recent characterization of a gene family (Slc26) encoding anion exchange proteins that accept a variety of monovalent and divalent substrates (4, 17, 27, 35). At least one-half of the 10 functional genes in this family have the ability to transport oxalate when functionally characterized in heterologous expression systems (1, 24, 27, 30, 31, 39), and several of these oxalate transporters are present in the intestine (1, 5, 8, 17, 18, 21, 27, 28, 30–32, 35, 36, 38). For example, Slc26a3 (DRA) and Slc26a6 (putative anion transporter [PAT1]) are expressed along the length of the alimentary system, with DRA being more abundant in apical membranes of large intestine (3, 16, 26, 35, 36) and PAT1 being localized to the apical membranes of villar enterocytes in the duodenum (35, 36, 38) and in acinar cells of the pancreas (23). Because PAT1 is relatively abundant in small intestinal enterocytes, it is an attractive candidate mediator for transepithelial oxalate transport in this intestinal segment, but this proposal has not been tested directly.

To establish the role of PAT1 in intestinal oxalate transport, we have compared ileal oxalate transport and urinary oxalate excretion in wild-type (WT) and Slc26a6 null [knockout (KO)] mice developed by targeted gene disruption (36). We report here that PAT1 is, indeed, an important component of vectorial oxalate transport in the mouse distal ileum as judged by several criteria. First, whereas WT mice show a small net ileal secretion of oxalate, tissues from PAT1 null mice exhibit large absorptive net oxalate fluxes. Second, 200 μM mucosal DIDS promoted net oxalate absorption in WT ileum by a reduction in the serosal-to-mucosal flux of oxalate but was without effect in the KO mouse ileum. Third, Cl⁻ removal from the mucosal media increased the net absorption of oxalate in both groups, but in PAT1 null mice net ileal oxalate absorption was two times that measured in the presence of luminal Cl⁻. Fourth, urinary oxalate excretion in PAT1 null mice was fourfold that of the WT. Based on these results, we propose that the PAT1 gene product in the mouse ileum mediates apical efflux of oxalate...
oxalate in exchange for Cl− and is therefore a significant component of the transcellular serosal-to-mucosal unidirectional oxalate flux.

MATERIALS AND METHODS

Animals. WT (Slc26a6+/+) and Slc26a6 null mice (Slc26a6−/−) were bred at the University of Cincinnati (Cincinnati, OH); the construction of the targeting vector and subsequent generation of the Slc26a6−/− mice (from the C57BL strain) is described elsewhere (36). All experimental procedures were conducted in accordance with protocols approved by the University of Florida and the University of Cincinnati Institutional Animal Care and Use Committees and following the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice of either gender were used in the transport experiments, and the mean body weights for all the WT mice used was 30.7 ± 5.2 g and that of all the KO mice was 30.9 ± 11.9 g. The mice always had free access to drinking water and food (LM-485; Harlan Teklad, Indianapolis, IN). Animals were killed by 100% CO2 followed by cervical dislocation.

Transepithelial transport measurements. The unidirectional and net fluxes of oxalate across isolated ileal segments were measured essentially as described previously (10, 12, 13, 15). Immediately after death and exsanguination of the mice, ~4 cm of the distal ileum was removed and thoroughly cleansed by flushing with ice-cold 0.9% NaCl. With the use of a dissecting microscope, remnant tags of connective tissue were removed, and the intestinal segment was opened along the mesenteric border. Flat sheets of tissue were mounted in modified Ussing chambers having an exposed tissue area of 0.34 cm2. Unidirectional fluxes of [14C]oxalate were measured across tissues bathed on both sides by 4 ml of buffered saline (pH 7.4) at 37°C circulated by vigorously bubbling with ice-cold 0.9% NaCl. With the use of an automated voltage clamp (VCCMC6; Physiologic Instruments, San Diego, CA). Typically, we have divided this total measurement time into two periods: an initial period (Per I) representing the average of the first three 15-min flux intervals (0–45 min) and a second period (Per II) representing the average of the last three 15-min flux intervals (60–105 min). The electrical parameters of the tissue were also recorded at 15-min intervals throughout the entire experiment. Tissue conductance (GT; mS/cm2) was calculated as the ratio of the open-circuit potential (Vt; mV) to the short-circuit current (Isc; μA/cm2) and net fluxes were computed on conductance-matched tissues pairs (GT within 15% of one another).

Urine collection. Mice were housed in metabolic cages and had free access to food and water; 24-h urine samples were made under mineral oil in vessels containing 10 μl of 2% sodium azide as a preservative (for pH, osmolarity, and Cl−) or in vessels containing 100 μl of 3.5 N HCl to ensure complete solubilization of crystallized oxalates.

Analytical methods. Urinary Cl− concentrations were determined with a chloridometer (Labconco, Kansas City, MO). Urinary osmolality was measured with a freezing point osmometer (Fiske Associates, Norwood, MA) and urine pH with a pH electrode (Accumet; Fisher Scientific, Philadelphia, PA). Creatinine was determined in the urine and serum samples using a modification of the Jaffé reaction as described previously (7). Oxalate was measured in both serum and urine using a coupled enzymatic (oxalate decarboxylase and formate dehydrogenase) assay procedure routine in our laboratory (9). Mouse blood collected by cardiac puncture was handled immediately with the appropriate precautions to prevent oxalogenesis, and serum pools from two to five mice were prepared for oxalate determination.

Fecal oxalobacter. Fecal samples were collected from mouse large intestine at the time tissue was being prepared for the flux studies. The presence of Oxalobacter sp. in the feces was determined by inoculating anaerobically sealed vials, containing 20 mM oxalate, with ~20 mg of fecal material. The potential loss of oxalate by microbial action in these vials at 37°C was determined 1 wk later by our routine enzymatic oxalate assay.

RNA isolation and real-time PCR. A 50- to 100-mg segment of the distal ileum from each mouse was preserved in RNA later (Ambion, Austin, TX), and total cellular RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Residual genomic DNA contamination was removed from the total RNA samples with the TURBO DNA-free kit (Ambion). Gene-specific oligonucleotide primers were designed with Primer3 (29) from murine reference nucleotide sequences retrieved from GenBank and are shown in Table 1. Real-time PCR was performed using the Quantitect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) with the DNA Engine Opticon continuous fluorescence detection system (MJ Research, San Francisco, CA). Briefly, 100 ng of total RNA was added to 25 μl of 2X Quantitect SYBR Green RT-PCR Master Mix,

### Table 1. Oligonucleotides designed from genes of interest and utilized for real-time PCR

<table>
<thead>
<tr>
<th>Gene (Alias)</th>
<th>Reference Sequence</th>
<th>Primer</th>
<th>3’-Oligo Sequence-3’</th>
<th>Position in Reference Sequence</th>
<th>Amplicon Size, bp</th>
<th>Amplicon Tm, °C</th>
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<tr>
<td>Slc26a1 (SAT1)</td>
<td>NM_174870</td>
<td>Forward</td>
<td>AGGTGCCAATACCCACAGAG</td>
<td>1239</td>
<td>216</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CTCGCGAGAGAGTGAAGAG</td>
<td>1454</td>
<td></td>
<td></td>
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<tr>
<td>Slc26a2 (DTDST)</td>
<td>NM_007885</td>
<td>Forward</td>
<td>ACCAAGAAGGTGGATTAGGA</td>
<td>3125</td>
<td>194</td>
<td>81.4</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GGAAGCTGCTGAAGCCAAATAC</td>
<td>3318</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slc26a3 (DRA)</td>
<td>NM_021353</td>
<td>Forward</td>
<td>TGCTGGGATGTGTGTCTTACA</td>
<td>5377</td>
<td>154</td>
<td>79.3</td>
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<td></td>
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<td>Reverse</td>
<td>GCGAGAGGCAAGCTGAATAT</td>
<td>690</td>
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<td>Slc26a6 (PAT1)</td>
<td>NM_134420</td>
<td>Forward</td>
<td>GGAATTGGAGCTGGACAGAG</td>
<td>160</td>
<td>161</td>
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<td></td>
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<tr>
<td>CFTR</td>
<td>NM_021050</td>
<td>Forward</td>
<td>GATCTGACCCAGCAAAGAGGTTTC</td>
<td>4715</td>
<td>230</td>
<td>78.6</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CACAGAGTGTAGTGGTCCACGTTCC</td>
<td>4944</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_001001303</td>
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<td>ACCAGAGGAGCTGGAGATGG</td>
<td>602</td>
<td>171</td>
<td>85.1</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CACATTTTGGCGTAAGAAC</td>
<td>772</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CFTR, cystic fibrosis transmembrane conductance regulator; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
250 ng of each gene-specific primer, and 0.5 μl of QuantiTect RT mix. The reactions were adjusted to 50 μl total volume with RNase-free water and incubated at 50°C for 30 min. RT was deactivated, and the HotStarTaq DNA polymerase was activated by incubation at 95°C for 15 min. Reactions were then subjected to 45 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Fluorescence data were collected after each extension step. After each PCR run, a melting-curve analysis was performed using the built-in software of the DNA Engine Opticon System to verify specificity of the RT-PCR product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference to normalize for amount of RNA added to the reverse transcription reaction. Data are presented as the difference in critical threshold cycles between the gene of interest and that of a reference gene $\Delta C(T)$ as previously described (22).

PAT1 immunoblots. Luminal membrane vesicles were prepared from mouse ileum according to established methods (35). Proteins (25 or 50 μg/lane) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk proteins and then incubated for 6 h with PAT1 antibody (35) at 1:500 dilution. The secondary antibody was a donkey anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL). The results were visualized using a chemiluminescence method (SuperSignal Substrate; Pierce) and captured on light-sensitive imaging film (Kodak, Rochester, NY).

Other solutions and chemical resources. [14C]oxalate (4.14 GBq/mmol) was purchased from Amersham (Piscataway, NJ). Concentrated stock solutions of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS; Molecular Probes, Eugene, OR) were prepared daily in DMSO. The final concentration of DMSO in the mucosal chamber after DIDS addition was <0.1%. Dibutylryl-cAMP (db-cAMP) and all other reagent-grade salts were purchased from Sigma-Aldrich (St. Louis, MO).

Statistical analyses. Comparisons between WT and KO mice were made using Student’s t-test (2-tailed, unpaired), whereas comparisons between Per I and Per II variates within a given experimental design were made by a paired t-test. Results are presented as means ± 1 SE, and differences between means were judged significant if $P < 0.050$. To compare differences in gene expression levels, $\Delta C(T)$ values were rank transformed before ANOVA.

RESULTS

Ileal oxalate transport in WT and KO mice. The isolated, short-circuited mouse ileum generally exhibited relatively constant electrical and transport characteristics for the duration of the present experiments. This stability is depicted in Fig. 1, which presents the unidirectional oxalate fluxes measured across adjacent ileal segments from a single KO mouse that were monitored in control buffers for 105 min. The subsequent sections presented in Fig. 1 represent comparisons between WT and KO mouse ileum obtained during the initial period (Per I). WT and KO mice had similar transepithelial conduc-

![Fig. 1. Comparison of oxalate transport across the isolated, short-circuited distal ileum from wild-type (WT) and putative anion transporter (PAT)-1 null mice [knockout (KO)]. In A, the unidirectional oxalate fluxes ($J_{OX}$) measured at 15-min intervals from a representative null mouse demonstrate the stability of tracer measurements up to 2 h. In B–F, the mean electrical and flux measurements for the first 3 intervals (0–45 min) of ileal tissues from WT mice (open bars, $n = 12$ tissue pairs from 7 animals) are compared with those from KO mice (filled bars, $n = 15$ tissue pairs from 10 animals). Tissue conductance ($G_T$) and short-circuit current ($I_{sc}$) are depicted in B and C, respectively. Unidirectional mucosal-to-serosal fluxes (M-S) of oxalate are presented in D and serosal-to-mucosal fluxes (S-M) of oxalate shown in E. The net flux for these conductance-matched tissues are presented in F. Error bars represent ±1 SE. Significant difference from the WT mouse. S-M, serosal to mucosal; M-S, mucosal to serosal.](http://ajpgi.physiology.org/doi/10.1152/ajpgi.00048.2006)
tances (Fig. 1B), but the \( I_{sc} \) measured in KO mouse ileum was significantly smaller (Fig. 1C). Under the conventions employed here, these positive \( I_{sc} \) values correspond to net electrogenic cation absorption (mucosal to serosal), net electrogenic anion secretion (serosal to mucosal), or some combination thereof.

The unidirectional and net fluxes of oxalate measured in WT and KO mouse ileum were markedly different (Fig. 1, D-F). The Slc26a6 null mice had a \( J_{sc}^{Ox} \) that was two times that measured in WT mice (Fig. 1D) and a \( J_{sc}^{Ox} \) that was approximately one-half that observed in the ileum from WT mice (Fig. 1E). These distinctions in unidirectional fluxes result in dramatic differences in net oxalate transport; WT mice exhibit a small net secretion, whereas KO mice strongly absorb oxalate (Fig. 1E). These results suggest that the PAT1 gene product, which from other studies appears to be exclusively located in the apical membrane of small intestinal villous enterocytes (36), may be more important in mediating oxalate secretion \( (J_{sc}^{Ox}) \) rather than mediating oxalate uptake from the luminal environment \( (J_{ms}^{Ox}) \).

Stilbene sensitivity of ileal oxalate transport. Oxalate transport pathways have long been known to be sensitive to disulfonic stilbenes (11, 19); consequently, we evaluated the effects of 200 \( \mu \)M DIDS, added to the mucosal compartment, on the unidirectional and net fluxes of oxalate across WT and KO mouse ileum. Mucosal DIDS had no significant effect on \( I_{sc} \) or \( G_T \) in either animal groups (data not shown). In WT mouse ileum, mucosal DIDS produced a reversal of \( J_{sc}^{Ox} \) (from \(-11.7 \pm 3.6 \) to \(17.6 \pm 9.1 \) pmol cm\(^{-2} \) h\(^{-1}\)) by a significant reduction (33\%) in \( J_{ms}^{Ox} \) and an increase in \( J_{ms}^{Ox} \) that was not statistically significant (Fig. 2A). In contrast to these observations on WT mouse ileum, mucosal DIDS had no effect on the unidirectional or net fluxes of oxalate in the KO mouse ileum (Fig. 2B).

The fact that oxalate transport is affected by mucosal DIDS in the WT ileum, but not the KO ileum, indicates that the PAT1 gene product is the principal DIDS-sensitive oxalate exchanger on the mucosal membrane, at least at 200 \( \mu \)M. Furthermore, the fact that mucosal DIDS significantly inhibited \( J_{sc}^{Ox} \), but not the absorptive unidirectional flux \( J_{ms}^{Ox} \), supports the notion that the apical exchanger of the ileal enterocyte that is stilbene sensitive (PAT1) mediates oxalate efflux from the cell to the lumen.

\( \text{Cl}^- \) dependence of ileal oxalate transport. Because the previous experiments suggested that PAT1 mediates oxalate efflux in the mouse ileum, we evaluated the possibility that mucosal \( \text{Cl}^- \) is a countertransport partner for PAT1-mediated oxalate efflux by removing mucosal \( \text{Cl}^- \). This maneuver generates bi-ionic diffusion potentials across the epithelium that contribute to the transepithelial potentials that must be clamped to establish the short-circuiting condition \((V_T = 0)\). Consequently, the electrical parameters measured in both WT (Fig. 3, B and C) and KO (Fig. 4, B and C) mice during Per I (0 mucosal \( \text{Cl}^- \)) are not a measure of active, electrogenic ion transport in the absence of mucosal \( \text{Cl}^- \). However, the unidirectional flux measurements presented in these figures can be compared with other flux measurements, since in all experiments the electrical driving forces are nominally clamped at \( V_T = 0 \) mV.

A representative flux experiment performed on adjacent pieces of ileum from a WT mouse is depicted in Fig. 3A, which shows that readmission of \( \text{Cl}^- \) to the mucosal buffer produced opposite effects on the unidirectional oxalate fluxes. \( \text{Cl}^- \) addition in Per II significantly decreased \( J_{ms}^{Ox} \) (17\%; Fig. 3D) and increased \( J_{ms}^{Ox} \) (25\%; Fig. 3E), which resulted in a small net secretion of oxalate (Fig. 3F). Note that the flux values observed in Per II are similar to those presented in Figs. 1 and 2, which supports the notion that \( \text{Cl}^- \) removal does not permanently impair oxalate transport in this preparation.

In the KO mouse ileum, \( \text{Cl}^- \)-free media produced changes in the electrical characteristics (Fig. 4, B and C) of the tissues that were similar to that observed in WT ileum, and the same constraints apply to interpretations of the significance of these observations. Oxalate fluxes measured on adjacent pieces of ileum from a KO mouse are shown in Fig. 4A in the absence and presence of \( \text{Cl}^- \). Unlike the WT ileum, where mucosal \( \text{Cl}^- \) altered both unidirectional oxalate fluxes, readmission of mucosal \( \text{Cl}^- \) only changed \( J_{ms}^{Ox} \), reducing it by 34\% (Fig. 3D).
was observed experimentally. Sides) at the end of Per I significantly increased of the apical membrane. The fact that in WT mucosal Cl− between Cl− mucosal Cl− both WT and KO mouse ileum. In WT tissues, the presence of important in mediating apical membrane oxalate exchange in
ments, then in KO mouse ileum lacking PAT1 this oxalate efflux pathway, as suggested in the DIDS experi-
23% reduction of $J_{\text{net}}^{Ox}$ (from 58.8 ± 11.9 to 35.9 ± 11.1 pmol·cm$^{-2}$·h$^{-1}$). In WT mice, although dB-cAMP did tend to reduce $J_{\text{net}}^{Ox}$ 16%, neither this, nor the other fluxes, was statistically different from Per I values. The observation that $J_{\text{net}}^{Ox}$ was not increased in either WT or KO mice suggests that PAT1-mediated oxalate efflux is not regulated by increases in cellular cAMP in mouse ileum.

**Ileal PAT1 protein and mRNA.** The absence of PAT1 protein and mRNA in Slc26a6 null mice was verified by immunoblot and real-time RT-PCR, respectively. As shown in Fig. 5, PAT1 protein was not detected in ileal luminal membrane homoge-
ates from Slc26a6−/− (KO) mice at 25 or 50 μg total protein but abundantly expressed in Slc26a6+/+ (WT) mice at both protein concentrations. Furthermore, we were unable to detect PAT1 mRNA in KO mouse ileum but did find it in the WT mice. These findings are in agreement with immunofluorescent labeling and mRNA expression patterns of PAT1 in the duo-
dena of KO and WT mice reported earlier (36).

**mRNA expression of other transporters.** Interpretation of the differences in oxalate transport between WT and KO mice is dependent on the assumption that the KO mouse is identical to WT with the exception of a single transport protein (PAT1). Clearly, compensatory adaptations of other exchangers could
confound interpretations of the present results, and it is equally apparent that it is not practical to validate this assumption for all possible transport or regulatory pathways. We have, however, considered how the absence of a functional Slc26a6 gene product affects the mRNA expression profiles of some other members of this gene family and cystic fibrosis transmembrane conductance regulator (CFTR). For each gene of interest, critical thresholds \[C(T)\text{gene}\] were determined from all ileal total RNA samples and normalized by subtracting the \[C(T)\] obtained for the reference gene, GAPDH. The resultant \(C(T)/H_9004\) is an inverse logarithmic expression of the abundance of a given mRNA; the higher the \(C(T)/H_9004\) the lower the quantity of a given mRNA. As shown in Table 2, there were no significant differences in the mRNA expression profiles between WT and KO mouse ileum for DRA, SAT1, DTDST, or CFTR as determined by real-time PCR, suggesting that these genes are not differentially expressed in the KO mouse ileum. It should be noted that, although comparisons between WT and KO mice for a given gene are valid, comparisons of relative mRNA abundance of different mRNAs is not necessarily suitable because PCR reaction efficiencies for different genes may not be identical. Consequently, we do not consider the differences in \(\Delta C(T)\) among different genes as a meaningful measure of their relative abundance.

Fig. 5. Absence of PAT1 protein in luminal membranes from ileum of KO mice. Immunoblot comparing luminal membrane homogenates from WT (Slc26a6\(+/-\)) and KO (Slc26a6\(-/-\)) mouse at loading concentrations of 25 and 50 \(\mu\)g/lane. Additional details are presented in MATERIALS AND METHODS.
ROLE OF PAT1 IN ILEAL OXALATE TRANSPORT

Table 2. Abundance of mRNA encoding several proteins possibly involved in oxalate transport or regulation across the distal ileum of WT and PAT1 KO mice as determined by real-time PCR

<table>
<thead>
<tr>
<th>Protein</th>
<th>WT</th>
<th>KO</th>
<th>ΔC(T)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>SAT1 (Slc26a1)</td>
<td>10.30±0.30</td>
<td>10.39±0.36</td>
<td>0.85</td>
<td>0.10</td>
</tr>
<tr>
<td>DTDST (Slc26a2)</td>
<td>4.54±0.07</td>
<td>5.22±0.34</td>
<td>2.54±0.42</td>
<td>0.06</td>
</tr>
<tr>
<td>DRA (Slc26a3)</td>
<td>2.29±0.29</td>
<td>24.0±0.37*</td>
<td>6.34±0.29</td>
<td>0.001</td>
</tr>
<tr>
<td>PAT1 (Slc26a6)</td>
<td>5.22±0.34</td>
<td>2.54±0.42</td>
<td>2.29±0.29</td>
<td>0.06</td>
</tr>
<tr>
<td>CFTR</td>
<td>2.29±0.29</td>
<td>24.0±0.37*</td>
<td>6.34±0.29</td>
<td>0.001</td>
</tr>
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WT, wild type; KO, knockout; ΔC(T) = C(T)gene - C(T)GAPDH. *PAT1 specific products were not detected in KO mice after 45 cycles of PCR. For purposes of statistical comparison, KO mice were arbitrarily assigned a C(T)PAT1 = 40.

Urinary oxalate excretion and serum oxalate. Because PAT1 is abundantly expressed in the renal proximal tubule, where it is proposed to have a role in transcellular Cl-/oxalate exchange (17, 18, 38), it was of interest to compare oxalate excretion patterns in WT and KO mice. To this end, we made duplicate to triplicate 24-h urine collections (over several weeks) on five WT and five KO mice. Of all the urinary parameters measured (Table 3), only urinary oxalate excretion was significantly different between the experimental groups, where KO mice were decidedly hyperoxaluric with respect to their WT littermates. Thus hyperoxaluria appears to be a phenotypic characteristic of PAT1 KO mice.

In another subset of animals, we measured serum creatinine levels and serum oxalate concentrations in pools of sera from two to five mice. There were no significant differences in serum creatinine or oxalate levels between WT and KO mice, which shows that, although the KO mice are hyperoxaluric, they are not hyperoxalemic under basal conditions.

Enteric oxalate-degrading bacteria. We also tested for the presence of oxalate-degrading bacteria in the large bowel of WT and KO mice, since a relative loss of these organisms in KO mice could conceivably increase the enteric oxalate burden and contribute to the observed hyperoxaluria in KO mice. However, no evidence was found for the presence of oxalate-degrading bacteria in either the WT or KO mouse large intestine.

DISCUSSION

The principal aim of the present study was to establish the physiological significance of a single anion exchanger (PAT1, Slc26a6) on oxalate transport across the isolated mouse ileum by comparing ileal oxalate fluxes in WT and Slc26a6 null mice. We found that ileal oxalate handling in WT mice is generally similar to that observed in rat and rabbit intestine in exhibiting Cl− dependence, stilbene sensitivity, and a net transport indicative of secondarily active transport systems (11). In contrast, net oxalate transport in the mouse ileum was not as dramatically altered by a cAMP analog as frequently observed in the other species (6, 11, 13, 15). Previous studies of oxalate transport across isolated, short-circuited epithelia (11–13) and isolated ileal brush-border membrane vesicles (19, 20) have implicated a variety of anion exchange systems as mediators of transmembrane oxalate flux. However, the apparent multiplicity of exchangers and possible exchange modes (2, 25, 27) has made it difficult to assign a specific function to any one exchanger. As shown here, studies of oxalate transport in animal models lacking specific candidate transporters (e.g., Slc26a family members) affords the opportunity to partition transcellular unidirectional oxalate fluxes into functional components.

PAT1 is expressed, in varying degrees, throughout the mouse gastrointestinal system, yet it is particularly abundant in the small intestine, a distribution that is the opposite of that of DRA (Slc26a3; see Ref. 35). Immunohistochemical staining of PAT1 in mouse duodenum labels the apical aspect of the villar, but not crypt, epithelium (35). In the duodenum, PAT1 functions as a apical Cl−/HCO3− exchanger mediating basal HCO3− secretion, as evidenced by reduced baseline HCO3− secretion in PAT1 null mouse duodenum and reduced Cl−/HCO3− exchange in duodenal brush-border membrane vesicles from KO mice (in the presence of an outward pH and HCO3− gradient) compared with WT mice (36). Slc26a6 has been shown to have an affinity for a variety of anions, including Cl−, oxalate, formate, sulfate, and HCO3−, and can function in a number of exchange modes involving different pairs of these ions (17, 35). In mouse duodenum, the Cl−/HCO3− exchange mode is important to HCO3− secretion, and it also appears, based on the present results, that PAT1 mediates Cl−/oxalate exchange in the ileum.

Model for ileal brush-border oxalate transport. Slc26a6+/− mice exhibit ileal oxalate transport characteristics that are decidedly different from those of their Slc26a6+/+ littermates. These observations, together with previous findings regarding the apical localization (36) and exchange modes of PAT1 (4, 17, 27, 38), suggest a working model (Fig. 6) of some components of oxalate transport across the brush-border membrane of ileal enterocytes. Under the conditions of the present experiments, we suggest that PAT1 mediates the efflux (cytosol to lumen) of oxalate across the apical membrane of ileal enterocytes in exchange for luminal Cl−. The first piece of evidence

Table 3. Comparison of selected urinary parameters and serum solutes in WT and PAT1 KO mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>KO</th>
<th>ΔC(T)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine Volume, ml/24 h</td>
<td>1.04±0.08</td>
<td>1.28±0.15</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Urine osmolarity, mosmol/l</td>
<td>2169±158</td>
<td>2129±215</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Urine pH</td>
<td>5.97±0.10</td>
<td>5.82±0.04</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Urinary oxalate excretion, μmol/24 h</td>
<td>131.8±13.2</td>
<td>173.7±20.5</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Urinary creatinine excretion, μmol/24 h</td>
<td>4.42±0.33</td>
<td>5.05±0.25</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Ox to Cr excretion ratio</td>
<td>1.31±0.19</td>
<td>5.07±0.57‡‡</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum creatinine, μmol/l</td>
<td>9.75±1.68</td>
<td>11.70±3.09</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum oxalate, μmol/l</td>
<td>23.52±2.12</td>
<td>30.21±4.22</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Means are based on the averages of repeated (duplicate or triplicate) urine collections for each mouse made in acidified (*) and nonacidified (‡) collections from WT (n = 5) and KO (n = 5) mice. Serum oxalate was measured in 5 WT serum pools and 6 KO serum pools (2–5 mice/pool). Serum creatinine was measured in WT (n = 8) and KO (n = 9) mice. ‡Significant difference compared with WT, P ≤ 0.05.
that oxalate transport by PAT1 could be replaced by a number of other intracellular anions, particularly $\text{HCO}_3^-$ generated by $\text{CO}_2$ production of the enterocyte. For example, in the mouse duodenum, PAT1 functions as an apical $\text{Cl}^-$/H$\text{CO}_3^-$ exchanger mediating the secretory flux of $\text{Cl}^-$/H$\text{CO}_3^-$ under basal conditions (35, 36). We have not tested this experimentally because reducing intracellular $\text{HCO}_3^-$ (by means of H$\text{CO}_3^-$-free buffers and carbonic anhydrase inhibitors) introduces uncertainties due to the existence of H$\text{CO}_3^-$-dependent transporters located at both poles of the epithelium that are affected by this maneuver.

The model presented in Fig. 6 assumes that the differences in oxalate transport between WT and KO mice originate solely from the lack of Slc26a6 in the KO mouse. However, it is always possible that there are compensatory adaptations of other transporters that directly or indirectly affect oxalate transport. For example, it has been reported that DRA (Slc26a3) is upregulated in Na$^+$/H$^+$ exchanger 3 null mouse colon (26). The overtwofold enhancement of $J_{\text{ms}}^{\text{Ox}}$ in PAT1 null mice (compared with WT; Fig. 1D) raised the possibility that this increase may be the result of an overexpression of the apical transporter mediating oxalate absorption. The molecular identity of the brush-border oxalate uptake pathway remains to be identified, but other members of the Slc26a family are possible candidates. In an earlier report, no differences in DRA (Slc26a3) mRNA were observed between WT and KO mouse small intestine (36), a result that we have confirmed here. In addition to our finding that DRA mRNA levels in WT and KO mouse ileum were similar, we found no evidence (Table 2) that CFTR or other members of the Slc26a family (SAT1, DRA, and DTDST) are upregulated in the KO mouse ileum. How other exchangers, like members of the Slc4 and Slc13 families, are involved in ileal oxalate transport and how they are impacted by PAT1 deletion needs to be assessed.

In the KO mouse ileum, $J_{\text{ms}}^{\text{Ox}}$ was significantly greater (2-fold) than that measured in WT ileum and, quantitatively, this elevation in $J_{\text{ms}}^{\text{Ox}}$ was mostly responsible for the significant net absorption of oxalate in the KO (Fig. 1) mouse ileum. If the exchangers mediating apical efflux (PAT1) and the unidentified apical influx exchanger(s) are located in the same cell, then the enhanced absorption of oxalate might be explained as a reduction in shunting (or recycling) of oxalate across the apical membrane in the KO mouse ileum. That is, in the absence of the PAT1 oxalate efflux pathway, more of the oxalate that is taken up by the apical influx pathway is taken up by the apical efflux pathway. Again, identification of the molecular nature of the apical exchanger(s) mediating oxalate uptake will be most useful in resolving these issues.

The model presented in Fig. 6 applies to the specific experimental conditions employed in our study; incorporation of additional transport elements not considered here may reveal alternative interpretations. For example, in sulfate-containing buffers, it is plausible that oxalate is also absorbed via oxalate-sulfate exchange, with luminal oxalate exchanging for cytosolic sulfate. The sulfate could then be recycled via the Na$^+$/SO$_4^2-$ cotransporter (25), which is abundantly expressed in the ileum of both WT and KO mice (data not shown). According to this scheme, in WT animals, the PAT1-mediated Cl$^-$/oxalate exchange would work in parallel with the oxalate/sulfate exchanger, again resulting in the recycling of oxalate with minimal net oxalate transport. Further studies are required to test these hypotheses.

Fig. 6. Schematic model of the possible contribution of PAT1 to oxalate (Ox) transport across the apical membrane of WT (top) and PAT1 KO (bottom) mouse ileum. Transport mechanisms at the basolateral membrane were not considered in this study; consequently, both basolateral uptake and efflux pathways for Ox are simply depicted by the bidirectional arrows at the serosal face of the epithelium. It is assumed that the represented transporters occur in a single cell type in the villus epithelium. In the WT cell, apical uptake is mediated by an unidentified exchanger that is not sensitive to 200 μM mucosal DIDS (Fig. 2), and efflux of Ox is mediated by PAT1 (labeled A6) that is DIDS sensitive. Both mechanisms are Cl$^-$/dependent; Cl$^-$ competes with Ox for the uptake mechanism, and Cl$^-$ is a countertransport partner on the PAT1 exchanger. In the WT cell, there is the potential for shunting Ox uptake (recycling) across the apical membrane through PAT1, thereby reducing the magnitude of net Ox absorption. In the KO model cell, only uptake mechanisms mediate apical Ox exchange, which remains insensitive to 200 μM mucosal DIDS (Fig. 2B). In the absence of the apical efflux mechanism in the KO mouse, Ox shunting across the brush border is reduced, and net Ox absorption is enhanced relative to WT (Figs. 1F, 2A, 2B, 3F, and 4F). See text for additional possibilities.
to evaluate the role of PAT1 in oxalate absorption under additional experimental conditions.

The results of the present study demonstrate that PAT1 mediates a significant fraction of oxalate efflux across the apical membrane of mouse ileum, which suggests that this specific avenue can contribute to net oxalate secretion by this intestinal segment. The observation of basal net oxalate secretion in WT mouse ileum agrees with previous findings of basal net oxalate secretion in the rat (14) and rabbit (6, 20) ileum under similar experimental conditions. In contrast to this phenomenon of basal net oxalate secretion in the small bowel, the distal colon of rats and rabbits typically exhibits basal net oxalate absorption (12, 15). As noted previously, the DRA protein is reported to be more abundant than PAT1 in the colon, whereas the inverse relationship applies to the small intestine (3, 26, 35, 36, 38). Whether the segmental differences in basal oxalate handling noted above can be simply attributed to differential expression of one or more anion exchangers remains to be established. Finally, it should also be noted that, when oxalate secretion is induced by secretagogues, transport avenues other than anion exchange mechanisms may predominate. For example, in rat and rabbit intestine, secretagogue-stimulated oxalate secretion exhibits many of the characteristics of cAMP-induced Cl– secretion (6, 11, 15).

Urinary oxalate excretion. The PAT1 gene product is also expressed in the proximal tubule where, like in the ileum (36), it is localized to the brush-border membrane (18). It is referred to as CFEX (Cl–/formate exchanger) in this tissue because it was originally suggested to represent the Cl–/formate exchanger (2, 17, 18, 38); however, CFEX is now known to have an affinity for several other anions, including oxalate and HCO3– and can function in a variety of exchange modes involving pairs of these anions (17, 35). A role for luminal oxalate in transepithelial NaCl reabsorption was suggested from earlier microperfusion studies which demonstrated that micromolar concentrations of luminal oxalate promoted a DIDS-sensitive increase in volume and Cl– reabsorption (2, 33, 34). Oxalate-stimulated increases in volume reabsorption were not observed in Slc26a6 null mice (36), further supporting a role for Cl–/oxalate exchange in proximal tubule function. Repeated determinations of urinary volume, osmolality, or Cl– excretion (Table 3) failed to reveal any significant differences between KO and WT mice, in agreement with studies performed on anesthetized mice (36). In addition, urine pH and urinary creatinine excretion were similar in the two groups as reported here. As noted elsewhere (36), downstream events may mitigate the expected salt-wasting effects resulting from a deficiency in oxalate-stimulated NaCl and volume reabsorption in the proximal tubule of KO mice.

In marked contrast, urinary oxalate excretion in KO mice was approximately fourfold greater than that of WT mice (Table 3). Whether this hyperoxaluric phenotype of the KO mouse is the direct result of an absence of Slc26a6 in renal epithelia, a consequence of the hyperabsorption of oxalate by the small intestine as observed here, or some combination of both, cannot be resolved from the present results. Serum oxalate levels were not significantly different between KO and WT mice, although there was a tendency toward hyperoxalemia (28% increase) in the KO mouse population studied here (Table 3). Remarkably, the degree (4×) of hyperoxaluria observed in KO mice vs. WT is similar to the degree of increase in urinary oxalate concentration reported for mice imbibing an oxalate precursor [1% ethylene glycol (EG)] for 4 wk (37) and comparable to that of rats drinking 0.75% EG for 2 wk (7, 10). This degree of hyperoxaluria in mice can lead to the formation of calcium oxalate crystals, the principal component of 75% of renal stones in humans; consequently, the Slc26a6 null mouse may also be useful as a model of nephrolithiasis that is independent of dietary manipulations.

In conclusion, our studies employing Slc26a6 null mice have identified, for the first time, a specific apical membrane protein involved in the transepithelial transport of oxalate. The PAT1 anion exchanger plays a significant role in the transepithelial secretory unidirectional flux of oxalate across the ileum by mediating oxalate efflux across the apical membrane. The hyperoxaluria observed in the PAT1 KO mice may be the result of enhanced ileal oxalate absorption or because of a defect in renal oxalate handling.

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