Parsing apical oxalate exchange in Caco-2BBe1 monolayers: siRNA knockdown of SLC26A6 reveals the role and properties of PAT-1

Robert W. Freel, Makoto Morozumi, and Marguerite Hatch

1Department of Pathology, Immunology, and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida; and 2Division of Urology, Faculty of Medicine, University of the Ryukyus, Nishihara, Okinawa, Japan

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Freel RW, Morozumi M, Hatch M. Parsing apical oxalate exchange in Caco-2BBe1 monolayers: siRNA knockdown of SLC26A6 reveals the role and properties of PAT-1. Am J Physiol Gastrointest Liver Physiol 297: G918–G929, 2009. First published August 27, 2009; doi:10.1152/ajpgi.00251.2009.—The purpose of this investigation was to quantify the contribution of the anion exchanger PAT-1 (putative anion transporter-1), encoded by SLC26A6, to oxalate transport in a model intestinal epithelium and to discern some characteristics of this exchanger expressed in its native environment. Control (Con) Caco-2BBe1 monolayers, 6–8 days postseeding, were compared with those transfected with a small interfering RNA targeted to SLC26A6 (A6KD). Radiotracer and Ussing chamber techniques were used to determine the transepithelial unidirectional fluxes of Ox2−, Cl−, and SO42− whereas fluorometric/BCECF measurements of intracellular pH were used to assess HCO3− exchange. PAT-1 was functionally targeted to the apical membrane, and SLC26A6 knockdown reduced PAT-1 protein (>60%) and mRNA (>75%) expression in A6KD. No net flux of Ox2−, Cl−, or SO42− was detected in Con or A6KD monolayers, yet the unidirectional fluxes in A6KD were reduced 50% compared with Con. The difference between Con and A6KD properties represents that mediated by PAT-1, and by this approach we found that PAT-1-mediated oxalate influx and efflux are inhibited equally by mucosal DIDS (EC50 ~5 μM) and that mucosal Cl− inhibits oxalate uptake with an EC50 < 20 mM. Transepithelial Cl− gradients supported large, DIDS-sensitive net absorptive or secretory fluxes of oxalate in a direction opposite that of the imposed Cl− gradient. The overall symmetry of PAT-1-mediated oxalate exchange suggests that vectorial oxalate transport observed in vivo is principally dependent on the magnitude and direction of counterion gradients.

Calcium oxalate nephrolithiasis is a relatively common disorder that may result from an imbalance in oxalate input (absorption from dietary sources and endogenous hepatic oxalogenesis) and oxalate output (renal excretion, intestinal secretion) (12). Intestinal epithelia play an important role in oxalate homeostasis by mediating bidirectional and net oxalate transport in a segment-specific manner (11, 12). Recent interest in identifying possible mediators of oxalate transport has been successfully focused on anion exchanger proteins encoded by the SLC26A family of genes (1, 3, 23). For example, the protein encoded by SLC26A6 (PAT-1, putative anion transporter-1; also referred to as CFEX, chloride-formate exchanger) has been shown to be an important mediator of oxalate secretion in the mouse ileum (10) and duodenum (13), since SLC26A6 knockout mice excrete less oxalate enterically and develop hyperoxaluria, which can promote stone formation. There are other anion exchangers that can participate in oxalate transport (11, 12) along the intestine that are also distributed segmentally and axially in a heterogeneous manner that may be targeted to the same membrane domain; hence it is difficult to establish the relative role of a single transporter like PAT-1 in animal models alone. One possible approach to resolve such issues would be to employ a simple, more homogeneous model of transepithelial oxalate transport by intestinal epithelia wherein gene silencing techniques might be applied to selectively partition the various exchange mechanisms.

In the present report, we present our studies concerning transepithelial oxalate (Ox2−) transport in Caco-2 monolayers in general and the specific role of PAT-1 to oxalate (and other anions) exchange as revealed by use of small interfering RNA (siRNA) techniques targeted to SLC26A6. Our general aims were twofold: 1) to establish the relative contribution of PAT-1 to transepithelial oxalate transport in a model system and 2) to determine some of the properties of PAT-1 such as inhibitor sensitivity and chloride ion affinity in its native environment. We found that Caco-2 cells do not support net anion transport, exhibiting symmetrical unidirectional fluxes of oxalate, chloride, and sulfate. Nor did a reduction of PAT-1 expression by siRNA induce any asymmetries in anion transport. Knockdown of SLC26A6 shows that PAT-1 mediates at least 50% of apical oxalate and 30% of apical chloride exchange symmetrically (influx and efflux); is strongly DIDS sensitive (EC50 ~ 5 μM); transports Ox2−, Cl−, HCO3−, and SO42− to a small degree; has an apparent Cl− affinity of less than 20 mM; and can mediate net secretion or absorption in the presence of a suitable transepithelial chloride gradient. On the basis of these findings we suggest that vectorial transport of oxalate mediated by PAT-1 is more dependent on the magnitude and direction of counterion driver gradients than an intrinsic property of the protein and that gene silencing is a useful tool in parsing apical anion exchange systems.

MATERIALS AND METHODS

Solutions and materials. The standard buffer contained the following (in mM): 140.8 Na+, 5.0 K+, 1.0 Mg2+, 1.0 Ca2+, 119.8 Cl−, 25.0 HCO3−, 1.0 SO42−, 1.6 HPO42−, 0.4 H2PO4−, 10.0 mannitol, 10.0 glucose. Gluconate was used as an anion replacement in chloride-free (Cl−-free), bicarbonate-free (HCO3−-free), and chloride-bicarbonate-free (Cl−-HCO3−-free) buffers. Calcium concentration was 6.0 mM in high-glucuronate solutions to compensate for gluconate chelation of Ca2+ (8) with adjustments in mannitol to balance osmolarities. Bi- carbonate solutions were gassed with humidified 95% O2-5% CO2. HCO3−-free solutions were gassed with humidified air, and all salines had a pH of 7.4 at 37°C. Oxalate was added to these buffers as Na2Ox...
at the time of experiment to total concentration of 1.5 μM including the contribution from radiolabeled oxalate.

J35ClO3, (mCi/g) and [35Cl]oxalate (115 mCi/mmol) were obtained from Amersham Biosciences (Piscataway, NJ) and N3SO42− (1,050 Ci/mmol) was purchased from Perkin Elmer (Boston, MA). The integrity of [35Cl]oxalate was routinely assessed enzymatically (9).

Nigericin, BCECF-AM [2’7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxyethyl ester], and DIDS (4,4’-diisothiocya-nothiobene-2,2’-disulfonic acid) were purchased from Molecular Probes/Invitrogen (Carlsbad, CA). All other chemicals were obtained through Sigma-Aldrich (St. Louis, MO).

**Cell culture and knockdown procedures.** We purchased the Caco-2 clone (C2BBe1, CRL-2102) from the American Type Culture Collection at passage 47. Cells were cultured in Eagle’s minimum essential medium (MEM) supplemented with 1-glutamine (2 mM), penicillin (120 IU/ml), streptomycin (120 μg/ml) from Mediatech (Manassas, VA), and 10% fetal bovine serum from Lonza (Walkersville, MD). Cells (between passage 51 and 66) were maintained at 37°C in a humidified environment of 5% CO2 in air. Culture medium was changed three times a week until monolayers reached ~90% confluency, at which time they were passaged and/or used for seeding experimental supports. For transport experiments, Caco-2 cells were seeded at high density (4 ×10^5 cells/cm²) on 12-mm Snapwell inserts (Corning, Lowell, MA) with 0.4 μm pore polycarbonate membranes, whereas for pH measurements, cells were seeded at high density on sterile, 15 mm round glass coverslips (Warner Instruments, Hamden, CT). Transepithelial resistance of monolayers in culture was routinely monitored using an Epithelial Voltohmmeter and chopstick electrodes from World Precision Instruments (Sarasota, Florida).

siRNAs for SLC26A6 (no. 30430) and a negative/scrambled control (AM4635) were purchased from Applied Biosystems/Ambion (Austin, TX). Caco-2 cells were transfected at the time of seeding by use of siLentFect Lipid Reagent from Bio-Rad (Hercules, CA). Prior to seeding, 50 μl of transfection solution (containing siRNA-lipid complexes dissolved in FBS-free MEM) was spotted on the upper face of a Snapwell membrane that had been equilibrated with standard culture media at 37°C in a CO2 incubator. Then 0.40 ml of a Caco-2 cell suspension in FBS-free MEM (10^5 cells/ml) was added to the upper face, yielding a final apical siRNA concentration of 30 nM. For most experiments, the apical solution was replaced 36–48 h later with standard MEM containing FBS. Transfections made in culture plates containing coverslips were similar, with the exception that only FBS-free MEM was used and the volumes were proportionally greater. Unless noted otherwise, experiments reported here were performed on monolayers 6–8 days after seeding and transfection. This transfection protocol was developed in pilot studies and provides for monolayers having high transepithelial resistance (>300 Ω·cm², corrected for support resistance) at a minimal siRNA concentration and at time points when siRNA functional effects persist in terms of anion transport and SLC26A6 message and protein expression.

**Transepithelial flux.** Transepithelial anion fluxes were measured across short-circuited Caco-2 monolayers grown on Snapwell inserts using EasyMount Ussing chambers and VCC MC6 voltage clamps from Physiologic Instruments (San Diego, CA). The voltage clamps were interfaced with the chamber solutions via 3 M KCl-agar bridges in contact with Ag-AgCl current and voltage electrodes. Each hemichamber contained 4.0 ml of buffered saline maintained at 37°C that was strongly circulated by gas lift systems in the chamber.

The standard protocol for measuring transepithelial anion flux was as follows. A radioisotope was added to either the serosal or mucosal reservoir (~0.5–2 μCi/chamber) 15 min after the cups were mounted in the diffusion chambers and sampling from the opposing reservoir began 10–20 min thereafter. At 15-min intervals for 105 min, a 1-ml sample was removed from the initially unlabeled reservoir and replaced with an identical, but isotope-free, buffer. At the beginning and end of each experiment a 50-μl sample of the labeled side was taken to determine the specific activity of the radioisotope. Samples were dissolved in 5 ml of Ecoscint A (National Diagnostics, Atlanta, GA) and isotopic activity was measured by liquid scintillation spectrometry with quench correction using a Beckman LS 6500 (Beckman Coulter, Fullerton, CA). Unidirectional anion fluxes [mucosal to serosal (JMS) and serosal to mucosal (JSM)] were computed from the changes in activity between successive samples with correction for dilution of the sampling reservoir and are presented as moles per square centimeter per hour. For statistical comparisons, the mean of the first three flux measurements (0–45 min) typically served as a control period (Per I) and the average of the last three flux measurements (60–105 min) represent the experimental period (Per II). Short-circuit current (Isc, μAmp/cm²) and transepithelial voltage (VT, mV) were also recorded at the 15-min intervals from which transepithelial resistance (Rt, Ω·cm²) or conductance (Gt, mS/cm²) were calculated by Ohm’s Law.

**Uptake experiments.** We measured oxalate flux in Caco-2 monolayers from the mucosal medium to the cell compartment (Jmuc) and from the serosal medium to the cell compartment (Jser) in the following manner. A Snapwell assembly (support with membrane cup) was removed from the culture environment, rinsed in bicarbonate-free buffer, and equilibrated at 37°C in the same buffer for 10–15 min before use. For uptake from the mucosal medium (apical influx), a six-well transport/culture plate containing 3.0 ml of the appropriate serosal medium in each well was placed on a heat block (37°C) on a reciprocal shaker. A Snapwell assembly was removed from the equilibration buffer, drained, and placed in a fresh well of the transport/culture plate. At time zero, 0.30 ml of radiolabeled buffer was added to the mucosal side and the reciprocal shaker was started (operating at 2 Hz). At a given time, uptake was stopped by removing the Snapwell assembly and vigorously and rapidly washing in four successive 400-ml ice-cold baths of bicarbonate-free buffer. The membrane filter was cut from the holder cup and placed in a scintillation vial. For uptake from the serosal medium (basolateral influx) the same procedure was used except that the serosal buffer contained the radiolabel and fresh warmed buffer was added to the mucosal compartment just before the Snapwell assembly was placed in a fresh well of the transport/culture plate. The membrane filters and 50-μl aliquots of the labeled media were dissolved in 5 ml of Ecoscint A and counted as described above.

**Intracellular pH.** Chloride-dependent bicarbonate exchange in Caco-2 monolayers was determined from measurements of intracellular pH (pHi). Confluent monolayers grown on coverslips were loaded with 5 μM BCECF-AM for 30 min at 37°C in standard buffer in a 5% CO2 environment followed by at least a 20-min period in dye-free buffer. The coverslip was positioned in a closed perfusion chamber (RC-20, Warner Instruments) that was mounted on a temperature-controlled holder (PH1, Warner Instruments) on the stage of a Nikon TE300 inverted microscope (Nikon Instruments, Melville, NY). The monolayer was continuously superfused by gravity (~1 ml/min) with warmed buffers gassed (95% O2-5% CO2) from 60-ml syringes, each reservoir being connected to a manifold with the perfusion chamber by low-CO2-permeable tubing and controlled by an electronic valve system (VC-6 System, Warner Instruments). Intracellular pH was calculated from fluorescence ratios measured every 10 s at excitation of 490/440 nm and emission at 530 nm by using a QuantMaster spectrofluorometer (Photon Technologies International, Birmingham, NJ) captured with a CoolSnap fx CCD camera (Photometrics, Tucson, AZ). Data acquisition and analysis were performed by use of ImageMaster software (PTI). A three-point calibration of the fluorescent signals was performed at the end of each experiment using the high-K+ concentration nigericin (10 μM) method (5, 24) in a solution having the following composition (in mM): 123 KCl, 1 MgSO4, 1 Ca gluconate, 5 HEPES, 50 mannitol, and 10 glucose, which was gassed with air and had a pH between 6.8 and 8.0.
Protein and RNA isolation. Total RNA was extracted from Caco-2 monolayers with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Residual genomic DNA contamination was removed with TURBO DNase (Ambion) for 1 h at 37°C, and DNase was removed from the RNA samples with DNase Inactivation Reagent (Ambion). DNA-free total RNA was quantified by measuring the absorbance at 260 and 280 nm (with A260/A280 ratios between 1.8 and 2.0 being considered acceptable) and stored at −80°C.

**Immunoblots.** Protein was precipitated from the organic phase generated from the RNA isolation step (above) by incubation with isopropanol for 10 min at room temperature. The protein precipitate was sedimented by centrifugation at 12,000 × g for 10 min and sequentially washed four times in 0.3 M guanidine hydrochloride in 95% ethanol followed by two washes in ethanol. Washing steps included sonication of the protein pellet, incubation at room temperature for 20 min and centrifugation at 7,500 × g for 5 min. The final protein pellet was resuspended in 5% SDS, and protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL). Total protein (200 μg) was separated on a 5% stacking-12% separating SDS-PAGE gel (Bio-Rad; Hercules, CA) by electrophoresis at 200 V for 30 min. Proteins were electrophoretically transferred to a Hybond ECL nitrocellulose membrane at 60 V for 3 h at 4°C. Membranes were blocked with 5% Blotto (Bio-Rad) in TBS-T (0.15 mM NaCl, 10 mM Tris, 0.1% Tween-20, pH 7.5) for 1 h. Polyclonal antibody to human PAT-1 (Alpha Diagnostic International; San Antonio, TX) was diluted 1:250 in 5% Blotto and incubated with membranes overnight at 4°C with gentle rocking. The membranes were washed six times for 5 min each in TBS-T. The membranes were then incubated for 30 min in 5% Blotto containing a 1:7,500 dilution of a donkey anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Amersham) followed by three subsequent 5-min washes in TBS-T and a final 5-min wash in TBS (TBS-T minus Tween-20). Blots were visualized by enhanced chemiluminescence (Pierce), exposure to Hyperfilm ECL (Amer- sham) followed by densitometric analysis using Image J. Membranes were stripped by incubation in Restore Western Blot Stripping Buffer (Pierce) for 20 min at 37°C. Membranes were reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described above with a 1:10,000 dilution of primary antibody (Ambion) for 1 h at room temperature and a 1:13,000 dilution of sheep anti-mouse IgG-HRP conjugate.

**Real-time RT-PCR.** Real-time RT-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 RNA was added to 25 μl of 2× QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Valencia, CA), 0.5 μM of each gene-specific primer (listed below) 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. Reverse transcriptases were deactivated and the HotStarTaq DNA polymerase was activated by incubation at 95°C for 15 min. Reaction tubes 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 at each extension step. After each PCR run, a melting curve analysis was performed by using the Opticon MONITOR software to verify the specificity of the RT-PCR product. In addition, all PCR runs also included the appropriate controls, including no-template controls to enable detection of contamination and no reverse transcriptase controls to test for contaminating genomic DNA. The Opticon MONITOR software was used to calculate the critical threshold value (Ct) for each sample, and the abundance of each target gene was analyzed by the 2−ΔΔCt comparative method (15, 20). GAPDH was used as an internal control and to normalize for the amount of RNA added to each reaction.

Oligonucleotide primers were designed by using Primer3 (21) with the NCBI reference nucleotide sequences for SLC26A6 (NM_022911) and GAPDH (NM_002046). Forward and reverse primers for SLC26A6 were (5′ → 3′) AGAAGCAGGAGCAGCT-GAAG and TCTACCTGCTCAGG, corresponding to starting positions of 1842 and 1991, respectively, and producing a PCR product of 150 nucleotides. Forward and reverse primers for GAPDH were (5′ → 3′) GAGTCAACGGATTTGGTCGT and GACAAGC-TCCCGTTCTCAG, corresponding to starting positions of 95 and 279, respectively, having a PCR product of 185 nucleotides.

**Statistics.** Comparisons between two means were made using Student’s t-test for paired or unpaired variates, as appropriate. Multiple comparisons were made by a one-way analysis of variance and Bonferroni’s post hoc test. Differences are considered significant if P ≤ 0.05. Results are presented as means ± 1 SE for n number of observations. All of the transport-related results were obtained by using monolayers from at least two separate seedings.

**RESULTS**

**SLC26A6 knockdown reduces PAT-1 mRNA and protein.** We differentially depressed the expression of SLC26A6 protein in Caco-2 cells by seeding at high density in the presence of an siRNA for SLC26A6 complexed to a lipid-based transfection agent. Each siRNA transfection was always accompanied by seeding of control monolayers and occasionally monolayers transfected with a scrambled siRNA (at the same concentration) serving as a negative control. The monolayers were not disturbed for up to 48 h, which facilitated the establishment of cell attachment and the subsequent development of a trans epithelial resistance suitable for transepithelial anion flux measurements. As shown in Fig. 1A, high-density seeding leads to a rapid development of RT in both siRNA-treated and control monolayers.

The extent and persistence of reduction in SLC26A6 mRNA in transfected Caco-2 cells, relative to nontransfected monolayers, was evaluated at several time points by real-time RT-PCR. As shown in Fig. 1B, the relative expression of SLC26A6 mRNA was reduced to 18% of nontransfected controls 2 days after seeding and transfection and was still 17% at day 6. However, 14 days after transfection the relative levels of SLC26A6 message were not significantly different from control monolayers. Parallel transfections using a scrambled siRNA as a negative control (Scram) demonstrated that reductions in SLC26A6 message were not due to a general effect of the silencing procedure employed since the relative expression of SLC26A6 mRNA in Scram was not significantly different from control monolayers at any time point examined (Fig. 1B).

SLC26A6 protein expression, relative to GAPDH, in 6-day Caco-2 monolayers was evaluated by immunoblot analysis. As shown in Fig. 1C, cells transfected with SLC26A6 siRNA exhibited a significant (~60%) reduction in PAT-1/GAPDH whereas scrambled siRNA did not affect protein expression.

These findings show that Caco-2 monolayers are amenable to the gene silencing techniques employed here and that at day 6 posttransfection mRNA and protein expression levels are significantly depressed to levels that should impact anion transport mediated by SLC26A6. Consequently, all of the following experiments were performed on 6- to 8-day-old Caco-2 monolayers.

**SLC26A6 knockdown reduces transepithelial oxalate, Cl− and SO42− fluxes.** To determine the impact of SLC26A6 knockdown on anion transport by Caco-2 cells we measured the unidirectional fluxes of [14C]oxalate and 36Cl− across control (Con), SLC26A6 knockdown (A6KD), and scrambled (Scram) monolayers under short-circuit conditions at 6–8 days post-
transfection. Figure 2 shows the results of experiments with Con and A6KD monolayers. Short-circuit current (Fig. 2A) and monolayer conductance (Fig. 2B) were constant for up to 105 min, and A6KD monolayers were not significantly different from Con monolayers. Control monolayers (Fig. 2C) exhibited constant unidirectional oxalate fluxes for up to 105 min and, importantly, mucosal-to-serosal oxalate flux \( (J_{SM}^{Ox}) \) was not significantly different from serosal-to-mucosal oxalate flux \( (J_{SM}^{Ox}) \). The average \( J_{SM}^{Ox} \) (pmol·cm\(^{-2}\)·h\(^{-1}\)) in Per I was 23.05 ± 2.42 and 24.38 ± 2.53 in Per II, whereas \( J_{SM}^{Ox} \) was 21.70 ± 2.73 in Per II and 23.57 ± 3.03 in Per II. The unidirectional oxalate fluxes in A6KD monolayers were significantly depressed 40% from the controls, clearly demonstrating that SLC26A6 makes an important contribution to transepithelial oxalate transport in Caco-2 monolayers. As with the Con monolayers, no net flux of oxalate was discernable in the A6KD monolayers (Fig. 2C); \( J_{SM}^{Ox} \) in Per I (pmol·cm\(^{-2}\)·h\(^{-1}\)) was 14.71 ± 2.20 and 15.50 ± 2.40 in Per II, whereas \( J_{SM}^{Ox} \) was 13.26 ± 1.76 in Per II and 14.93 ± 1.84 in Per II. In parallel experiments, oxalate transport in Caco-2 monolayers transfected with scrambled siRNA (Scram, \( n = 9 \)) was similar to that of control monolayers in the standard bicarbonate buffer: In Per I, \( J_{SM}^{Ox} = 21.4 ± 1.3 \) and \( J_{SM} = 22.1 ± 0.7 \).

A similar series of studies was performed using 36Cl\(^{-} \) to establish the contribution of SLC26A6 to transepithelial chloride transport in Caco-2 monolayers in standard bicarbonate buffer. As shown in Fig. 2D, unidirectional chloride fluxes in Con were constant and not significantly different. The average mucosal-to-serosal chloride flux \( (J_{SM}^{Cl}, \mu \text{mol·cm}^{-2}·\text{h}^{-1}) \) in Per I was 3.95 ± 0.23 and 4.12 ± 0.29 in Per II, whereas serosal-to-mucosal chloride flux \( (J_{SM}^{Cl}) \) was 4.28 ± 0.39 in Per II and 4.10 ± 0.29 in Per II. In the A6KD monolayers, no significant net Cl\(^{-} \) flux was observed, but the unidirectional fluxes were ~75% of Con. Thus, in Per I, \( J_{SM}^{Cl} \) (pmol·cm\(^{-2}\)·h\(^{-1}\)) of A6KD monolayers was 3.25 ± 0.17 and 3.16 ± 0.20 in Per II, whereas \( J_{SM}^{Cl} \) was 3.48 ± 0.18 in Per II and 3.39 ± 0.16 in Per II. In both Con and A6KD monolayers we frequently observed that \( J_{SM}^{Cl} \) was slightly (0.10–0.30 \( \mu \text{mol·cm}^{-2}·\text{h}^{-1} \)) larger than \( J_{SM}^{Cl} \), but these differences were not significant statistically. The small \( I_{sc} \) recorded in Caco-2 monolayers \((-0.17 ± 0.01 \mu \text{eq·cm}^{-2}·\text{h}^{-1}) \), Fig. 1A Per I) in standard buffer is consistent in sign and magnitude with this small amount of net chloride secretion. In Cl\(^{-}\)-free solutions, the \( I_{sc} \) in Con and A6KD monolayers is significantly reduced to \(-0.038 ± 0.006\) and \(-0.026 ± 0.004\), respectively, \( n = 12 \), further suggesting that the \( I_{sc} \) in Caco-2 cells is a chloride secretory current.

The contribution of SLC26A6 to transepithelial sulfate transport was also examined. As shown in Fig. 3, no net transport of sulfate by Con or A6KD monolayers was detected. Although transfection with siRNA for SLC26A6 produced a statistically significant reduction in mucosal-to-serosal \( (J_{SM}^{SO}) \) and serosal-to-mucosal sulfate flux \( (J_{SM}^{SO}) \), the extent of reduction was small (15–20%) compared with that of oxalate and chloride. (The knockdown of oxalate transport in this series was similar to that presented throughout this report, so the...
sulfate results were not due to a lower knockdown efficiency). However, mucosal DIDS (100 μM) more strongly inhibited both unidirectional SO$_4^{2-}$ fluxes (>95%) when compared with Cl$^-$ or Ox$^{2-}$ fluxes. These observations suggest that most sulfate transport in Caco-2 cells occurs via the transcellular pathway and that SLC26A6 has a relatively minor role in SO$_4^{2-}$/HCO$_3^-$ transport.

The results presented in Figs. 2 and 3 clearly show that in Caco-2 monolayers 1) anion fluxes are constant through time and 2) do not exhibit net transport and 3) a significant portion of the transepithelial flux of Ox$^{2-}$ and Cl$^-$ is mediated by SLC26A6.

**SLC26A6 knockdown reduces Cl$^-$-dependent HCO$_3^-$ exchange.** Since chloride-bicarbonate exchange is an expected and prominent transport mode of SLC26A6 (1, 14, 23), we evaluated the impact of SLC26A6 knockdown on Cl$^-$-dependent HCO$_3^-$ efflux in control, scrambled siRNA-treated, and SLC26A6 siRNA-treated Caco-2 cells (Fig. 4). When superfused with standard buffer, intracellular pH for Con ($n = 8$) and Scram ($n = 7$) monolayers (7.34 ± 0.03 and 7.29 ± 0.04, respectively) were not significantly different; however, A6KD ($n = 8$) monolayers were slightly, but significantly ($P < 0.02$, more alkaline (pHi = 7.45 ± 0.28) under control conditions. As shown in Fig. 4, superfusing with Cl$^-$-free HCO$_3^-$ buffer results in an alkalinization of the cytosol in all cases, owing, at least in part, to bicarbonate accumulation resulting from the reduction of Cl$^-$-driven HCO$_3^-$ efflux and influx. The average, steady-state change in pHi values (baseline minus Cl$^-$-free) for Con and Scram were significantly greater than that observed in A6KD monolayers. Readdition of chloride to the superfusate resulted in a return of pH$_i$ to basal levels in all conditions. As shown in the insets in Fig. 4, the rates of alkalinization and recovery (8 pH$_i$/min calculated as the slope of six points collected in the first minute after superfusate Cl$^-$ was changed) in A6KD monolayers was significantly smaller (<50%) than those in Con and Scram monolayers. These results indicate that Caco-2 cells exhibit Cl$^-$-dependent HCO$_3^-$ exchange that is reduced significantly when SLC26A6 protein expression is reduced by siRNA.
SLC26A6 knockdown reduces apical oxalate uptake. The marked symmetry of anion fluxes, even when PAT-1 is reduced, is an interesting finding, if SLC26A6 is properly targeted to the apical membrane. One possible explanation for this symmetry is that the SLC26A6 protein is targeted to the both the apical and basolateral membrane of Caco-2 cells. To functionally test this possibility we compared the apical uptake of [14C]oxalate in Con and A6KD monolayers with that of basolateral oxalate uptake in Con and A6KD monolayers. If the SLC26A6 protein is targeted to both membranes, then oxalate uptake from both apical and basolateral sides of A6KD monolayers would be reduced compared with Con. Figure 6 presents the time course of [14C]oxalate uptake in 6- to 8-day-old Caco-2 monolayers in bicarbonate-free buffers at 37°C. To increase the magnitude of oxalate uptake, the cis-side buffer was Cl⁻-HCO₃⁻ free buffer, and the trans-side buffer was HCO₃⁻ free. As shown in Fig. 6A, silencing SLC26A6 results in a significant reduction in the rate of oxalate accumulation from the apical side, whereas accumulation from the basolateral side (Fig. 6B) was the same for Con and A6KD monolayers. Uptake from the serosal aspect was more rapid than from the mucosal side, yet the equilibrium value of oxalate uptake (∼1.5 pmol·cm⁻²) was independent of loading side. Comparison of initial, first-order uptake rates summarized in Fig. 6C for the four sets of monolayers plainly shows SLC26A6 silencing only affects [14C]oxalate uptake across the apical membrane in A6KD monolayers. We conclude that the SLC26A6 protein is predominantly, if not fully, targeted to the apical membrane in Caco-2 cells.

Dose response of SLC26A6 to mucosal DIDS. Addition of DIDS to the mucosal saline reduced the unidirectional Ox²⁻ and Cl⁻ fluxes across short-circuited Caco-2 monolayers in standard bicarbonate buffer. As shown in Fig. 7A, mucosal DIDS reduced Jₘₕ and Jₘ₃ similarly in time and magnitude, with the full depression occurring only after 30 min. Mucosal DIDS decreased Jₘ₅ 72% in Con monolayer (from 23.2 ± 1.0

This indicates that chloroform or bicarbonate exert a dominant effect on oxalate transport in Caco-2 monolayers, not as required countertransport species (e.g., a trans effect) but as competitors with oxalate (e.g., a cis effect) for binding sites on the exchange proteins.

and intracellular buffering systems in Caco-2 cells that could contribute to the overall recovery of pH, following Cl⁻ readmission; however, the fact the recovery rates in A6KD monolayers were so strongly affected indicates that SLC26A6 contributes considerably to Cl⁻-HCO₃⁻ exchange in these cells.

Anion dependence of oxalate fluxes in control and SLC26A6 knockdown monolayers. Symmetrical replacement of the principal anions in the standard buffer produced significant increases in the unidirectional oxalate fluxes in both Con and A6KD monolayers measured in Per I. As shown in Fig. 5, in Con monolayers (open bars) bicarbonate removal increased Jₘ₅ in Per I by ~48% whereas Cl⁻ removal increased Jₘ₅ 311%. A6KD (shaded bars) monolayers responded similarly: Jₘ₅ increased 69% in HCO₃⁻-free buffers and 282% in Cl⁻-free buffers in Per I. Oxalate fluxes in the A6KD monolayers were significantly smaller than their corresponding values in Con monolayers; however, only in the Cl⁻-free condition were oxalate fluxes significantly greater than the values measured in standard buffer for either Con or A6KD. Symmetric sulfate replacement in the standard buffer produced no significant changes in oxalate transport or the electrical characteristics of Con or A6KD monolayers (not shown).

The stilbene sensitivity of Jₘ₅ in the absence of Cl⁻ or HCO₃⁻ was also assessed by adding 100 μM DIDS to the mucosal compartment and measuring oxalate flux in Per II. The DIDS-insensitive components of Jₘ₅ for each condition are indicated by the darkened section at the base of each bar in Fig. 5. These components of Jₘ₅ were not significantly different from that measured in Con with the standard buffer, which suggests that the DIDS-insensitive flux of oxalate principally represents paracellular oxalate transport.

These results indicate that chloride and bicarbonate exert a dominant effect on oxalate transport in Caco-2 monolayers, not as required countertransport species (e.g., a trans effect) but as competitors with oxalate (e.g., a cis effect) for binding sites on the exchange proteins.

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SLC26A6 knockdown reduces apical oxalate uptake. The marked symmetry of anion fluxes, even when PAT-1 is reduced, is an interesting finding, if SLC26A6 is properly targeted to the apical membrane. One possible explanation for this symmetry is that the SLC26A6 protein is targeted to the both the apical and basolateral membrane of Caco-2 cells. To functionally test this possibility we compared the apical uptake of [14C]oxalate in Con and A6KD monolayers with that of basolateral oxalate uptake in Con and A6KD monolayers. If the SLC26A6 protein is targeted to both membranes, then oxalate uptake from both apical and basolateral sides of A6KD monolayers would be reduced compared with Con. Figure 6 presents the time course of [14C]oxalate uptake in 6- to 8-day-old Caco-2 monolayers in bicarbonate-free buffers at 37°C. To increase the magnitude of oxalate uptake, the cis-side buffer was Cl⁻-HCO₃⁻ free buffer, and the trans-side buffer was HCO₃⁻ free. As shown in Fig. 6A, silencing SLC26A6 results in a significant reduction in the rate of oxalate accumulation from the apical side, whereas accumulation from the basolateral side (Fig. 6B) was the same for Con and A6KD monolayers. Uptake from the serosal aspect was more rapid than from the mucosal side, yet the equilibrium value of oxalate uptake (∼1.5 pmol·cm⁻²) was independent of loading side. Comparison of initial, first-order uptake rates summarized in Fig. 6C for the four sets of monolayers plainly shows SLC26A6 silencing only affects [14C]oxalate uptake across the apical membrane in A6KD monolayers. We conclude that the SLC26A6 protein is predominantly, if not fully, targeted to the apical membrane in Caco-2 cells.

Dose response of SLC26A6 to mucosal DIDS. Addition of DIDS to the mucosal saline reduced the unidirectional Ox²⁻ and Cl⁻ fluxes across short-circuited Caco-2 monolayers in standard bicarbonate buffer. As shown in Fig. 7A, mucosal DIDS reduced Jₘ₅ and Jₘ₃ similarly in time and magnitude, with the full depression occurring only after 30 min. Mucosal DIDS decreased Jₘ₅ 72% in Con monolayer (from 23.2 ± 1.0
in Per I to 6.5 ± 1.9 pmol·cm⁻²·h⁻¹ in Per II) and the changes in $J_{SM}^{\text{Ox}}$ were the same (Fig. 7A). In A6KD monolayers both unidirectional oxalate fluxes were reduced 48% compared with Con in Per I and DIDS reduced both fluxes to values that were not significantly different from Con in Per II. Thus in this series both Con and A6KD monolayers exhibited a DIDS-insensitive oxalate flux, $J_{SM}^{\text{Ox}}$, which, as noted previously, likely represents paracellular flux. Unidirectional chloride fluxes exhibited a similar pattern in response to mucosal DIDS (Fig. 7B). In this case, unidirectional chloride fluxes in A6KD monolayers were 73% of Con cells and mucosal DIDS reduced both to ~2.0 pmol·cm⁻²·h⁻¹ in Per II. At a concentration of 100 μM, mucosal DIDS inhibits $J_{SM}^{\text{Cl}}$ maximally. The difference between Con and A6KD monolayers represents

We performed a more detailed analysis of DIDS inhibition of anion exchange in Caco-2 cells with the expectation that a comparison of the dose-response relations between Con and A6KD monolayers would reveal (by difference) the dose response of SLC26A6 alone. The experimental protocol was to measure flux in the absence of DIDS (Per I) and then in the presence of a particular mucosal concentration of DIDS in Per II. To emphasize the degree of knockdown, the results for A6KD monolayers have been presented as a function of the mean Per I values for the Con monolayers in this series ($J_{SM}^{\text{Ox}} = 25.6 ± 1.3$ pmol·cm⁻²·h⁻¹, $n = 42$). As shown in Fig. 8A, addition of 0.1 to 250 μM DIDS to the mucosal solution reduced $J_{SM}^{\text{Ox}}$ in a dose-dependent manner in both Con (○) and A6KD monolayers (●). The solid lines represent sigmoidal dose-response curves fit to these data using nonlinear regression analysis (SigmaPlot 10). Presented in this fashion, it is clear that 1) $J_{SM}^{\text{Ox}}$ in A6KD monolayers is ~50% of Con, 2) ~20% of $J_{SM}^{\text{Ox}}$ in the control monolayers is DIDS insensitive, and 3) 100 μM mucosal DIDS inhibits $J_{SM}^{\text{Ox}}$ maximally. The difference between Con and A6KD monolayers represents

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**Fig. 6.** Time course of oxalate accumulation across the apical membrane (A) and basolateral membrane (B) for Con and A6KD monolayers in bicarbonate-free buffers. Apical uptake was determined with a Cl⁻-HCO₃⁻-free mucosal buffer and a HCO₃⁻-free serosal buffer, whereas the basolateral uptake measurements were made with the buffer compositions reversed. Each point represents the mean of 6–8 measurements and the error bars represent ±1 SE. *Significant difference between Con and A6KD.

**Fig. 7.** DIDS inhibition of unidirectional anion fluxes across Caco-2 monolayers in standard bicarbonate buffer. Open symbols in this figure represent Con monolayers, solid symbols signify A6KD monolayers; circles represent $J_{SM}^{\text{MS}}$ (MS) and squares $J_{SM}^{\text{SM}}$ (SM). A: time course of mucosal DIDS (100 μM) inhibition of [¹⁴C]oxalate transport across short-circuited Con monolayers ($n = 7$) and A6KD ($n = 9$) monolayers. B: time course for [³⁶Cl⁻] fluxes measured across Con ($n = 7$) and A6KD ($n = 7$) Caco-2 monolayers. Current and conductance were not significantly altered by mucosal DIDS. Error bars represent ±1 SE.
the dose-response relation for SLC26A6 protein expressed in the mucosal membrane of Caco-2 cells and, as shown in Fig. 8A (dashed line), it exhibits a marked sensitivity to mucosal DIDS (EC50 = 4.4 ± 1.3 μM). Figure 8B depicts the DIDS sensitivity of PAT-1 mediated oxalate efflux across the apical membrane measured as jOxM in Con and A6KD monolayers as discussed above. The EC50 for serosal-to-mucosal oxalate flux mediated by PAT-1 (7.5 ± 1.4 μM) was similar to that observed for the opposing flux (Fig. 8A), which demonstrates that DIDS symmetrically inhibits apical influx and efflux mediated by PAT-1 in Caco-2 cells.

In the same manner we examined the DIDS sensitivity of mucosal-to-serosal chloride fluxes in Con and A6KD monolayers at 6–8 days postseeding in standard bicarbonate buffer. As shown in Fig. 8C, jClM decreased with increasing mucosal DIDS in both Con and A6KD Caco-2 cells. Again, the difference between these two relationships (dashed line) represents the contribution of the SLC26A6 exchanger to apical chloride exchange and exhibits an EC50 = 8.1 ± 1.4 μM. Although there is a significant difference in the ability of 100 μM mucosal DIDS to reduce transepithelial oxalate and chloride transport (Fig. 7), this stilbene appears to block SLC26A6-mediated Ox− flux to the same degree (EC50 between 5 and 8 μM DIDS).

Chloride dependence of SLC26A6-mediated oxalate flux. As shown previously (Fig. 5), oxalate transport is significantly increased in the absence of the major anions like Cl− in both Con and A6KD monolayers. Since the preceding experiments show that the differential depression of SLC26A6 in Caco-2 cells provides a convenient means to define the properties of this exchanger, we sought to establish the chloride dependence of SLC26A6-mediated jOxM using this approach. As shown in Fig. 9, mucosal chloride concentration strongly affects jOxM in both Con and A6KD monolayers with half-maximum effects occurring at ~20 mM mucosal chloride in both cases. The inset in Fig. 9 presents the contribution of SLC26A6 exchanger to jOxM in Caco-2 cells. Here, the differences between Con and A6KD monolayers (= PAT-1 component) at each mucosal Cl− concentration have been plotted as a percentage of the maximal SLC26A6-mediated flux (measured in 0 mM Cl−). The solid line in the inset represents a fit to the data points by a one-site ligand binding model (SigmaPlot 10) yielding an EC50 of 18.7 ± 3.3 mM Cl−.

Transepithelial chloride gradients generate large net oxalate fluxes. The fact that Caco-2 monolayers at 6–8 days postseeding do not support a net transport of oxalate (Fig. 2C) or chloride (Fig. 2D) when bathed by symmetrical buffers...
suggests the absence of meaningful gradients for driver ions (countertransport partners like Cl\(^-\) and/or HCO\(_3\)\(^-\)) in these conditions. However, imposing a transepithelial gradient for a counterion should produce sufficient asymmetries at either membrane (depending on the orientation of the gradient) to generate a net transepithelial oxalate flux. To test this hypothesis we measured the unidirectional oxalate fluxes in Con and A6KD monolayers having an imposed transepithelial Cl\(^-\) gradient using the standard two-period protocol. The unidirectional fluxes of oxalate in the absence of mucosal chloride were strikingly asymmetric as shown in Fig. 10. In control monolayers (Fig. 10A) during Per I, \(J_{\text{ON}}\) averaged 164.1 ± 9.2 pmol·cm\(^{-2}\)·h\(^{-1}\), \(J_{\text{SM}}\) was 9.9 ± 2.3 pmol·cm\(^{-2}\)·h\(^{-1}\), resulting in a net oxalate flux (\(J_{\text{net}}\)) of 154.2 ± 14.1 pmol·cm\(^{-2}\)·h\(^{-1}\).

Addition of 100 μM DIDS to the mucosal side decreased \(J_{\text{MS}}\) to 11.3 ± 1.7 and \(J_{\text{SM}}\) to 2.7 ± 0.4 pmol·cm\(^{-2}\)·h\(^{-1}\), resulting in a small but significant (\(P < 0.001\)), DIDS-insensitive net oxalate flux of 8.6 ± 1.6 pmol·cm\(^{-2}\)·h\(^{-1}\). A6KD monolayers (Fig. 10B) exhibited a smaller \(J_{\text{ON}}\) of 65.0 ± 3.6 pmol·cm\(^{-2}\)·h\(^{-1}\) in Per I because \(J_{\text{MS}}\) was less than half (73.3 ± 3.6 pmol·cm\(^{-2}\)·h\(^{-1}\)) that of control monolayers. As with the Con monolayers, DIDS strongly reduced \(J_{\text{MS}}\), leaving a small, significant (\(P = 0.012\)) net flux of oxalate in Per II of 4.5 ± 1.4 pmol·cm\(^{-2}\)·h\(^{-1}\).

Imposition of a serosal-to-mucosal chloride gradient by using Cl\(^-\) and HCO\(_3\)\(^-\)-free serosal buffers and HCO\(_3\)\(^-\)-free mucosal buffers produced net secretory fluxes of oxalate that were persistent and DIDS sensitive (Fig. 11), effectively mirroring the previous results (Fig. 10).

In this case net secretory fluxes of oxalate in Con and A6KD monolayers of 154.8 ± 8.9 and 59.7 ± 10.3 pmol·cm\(^{-2}\)·h\(^{-1}\), respectively.

Together, the results presented in Figs. 10 and 11 show that 1) gradient-driven net absorptive or secretory oxalate fluxes are mostly DIDS sensitive and, therefore, mostly transcellular, 2) SLC26A6 mediates at least 50% of absorptive or secretory flux of oxalate, and 3) transepithelial Cl\(^-\) gradients very strongly increase the unidirectional oxalate flux in the opposing direction, while having a much smaller impact on oxalate fluxes in the same direction.

**DISCUSSION**

Many of the conclusions presented in this report are based on transepithelial flux measurements that include apical and basolateral components in parallel with paracellular components. Possible complications arising from paracellular and/or basolateral transport by studying transepithelial anion flux are mitigated by two facts. First, we have found no evidence that might suggest that the basolateral membrane exerts any significant rate limitations on transepithelial oxalate transport (Figs. 2, 3, 6, 7). Second, any complication imposed by the presence of additional transport barriers in parallel or in series with the apical membrane would be expected to exhibit the same effects in both Con and A6KD monolayers. Hence the difference between Con and A6KD cells represents the contribution of SLC26A6 alone. Since residual PAT-1 likely contributes to anion exchange measured after silencing, the results considered below represent the minimum contribution of PAT-1 to apical anion exchange in Caco-2 cells.

**SLC26A6-mediated bicarbonate transport.** Chloride-bicarbonate exchange is one of the principal exchange modes of SLC26A6, being observed in mouse (14, 22, 26) and human (6) orthologs, and as such it would be expected that this modality would be reduced after silencing of SLC26A6 in Caco-2 cells. We measured Cl\(^-\)-HCO\(_3\)\(^-\) exchange rates by measuring intracellular pH changes imposed by removal and readdition of Cl\(^-\) in Caco-2 cells superfused with HCO\(_3\)/CO\(_2\) buffers. The rate of pHi recovery on readmision of Cl\(^-\) in A6KD cells was about half that observed in Con and negative controls (Scram), indicating that SLC26A6 does indeed contribute to the regulation of intracellular pH operating in a Cl\(^-\)-HCO\(_3\)\(^-\) exchange modality. Although we did not estimate intracellular buffering capacity in these experiments, the dramatic depression observed in A6KD monolayers alone would be difficult to explain as a simple difference in buffering capacity and is sufficient qualitative verification, independent of radiotracer methodologies, that SLC26A6 silencing depresses Cl\(^-\)-HCO\(_3\)\(^-\) exchange.

**SLC26A6-mediated sulfate transport.** Transepithelial sulfate fluxes were symmetrical in Con and A6KD monolayers and were strongly inhibited by 100 μM mucosal DIDS (Fig. 4). What is most interesting is the fact that knockdown of SLC26A6 only reduced transepithelial SO\(_4\)\(^2\)\(^-\) fluxes by 17%, but 100 μM mucosal DIDS inhibited total sulfate transport by 96% and that of SLC26A6-mediated transport by 94%. SLC26A6-mediated SO\(_4\)\(^2\)\(^-\) exchange has been reported for the mouse (6, 14, 22, 27) and in some (16), but not all (6, 25), studies of the human ortholog. Although there is vigorous and symmetric sulfate transport across Caco-2 cells that is strongly
DIDS inhibitable, the SLC26A6-mediated component contributes only a small fraction to transepithelial sulfate transport. These findings suggest that paracellular $\text{SO}_4^{2-}$ flux is small (DIDS insensitive) and the bulk of transepithelial $\text{SO}_4^{2-}$ flux is mediated by a system other than SLC26A6. A highly DIDS-sensitive $\text{SO}_4^{2-}$ transport system has been observed in Caco-2 cells, and this transport activity was postulated to be mediated by the Downregulated in adenoma (DRA) protein encoded by SLC26A3 (2). DRA-mediated sulfate transport has also been reported for other experimental systems with variable but generally low DIDS sensitivity (17, 18), and DRA-mediated $\text{Cl}^{-}$ uptake by Caco-2 cells exhibited half-maximal effect around 100 $\mu$M DIDS (19). Although a DRA exchanger would be a logical candidate to account for non-SLC26A6-mediated sulfate fluxes in our study, the strong DIDS sensitivity exhibited by sulfate transport observed here (Fig. 4) precludes a definitive answer. We have also detected mRNA of SLC26A2 Diastrophic dysplasia sulfate transporter (DTDST) in Caco-2 monolayers (unpublished observations), and human and rat orthologs of DTDST have been reported to mediate robust $\text{SO}_4^{2-}$ transport when expressed in Xenopus oocytes (6).

**DIDS inhibition of PAT-1-mediated oxalate exchange.** The disulfonic stilbene DIDS is the prototypical inhibitor of anion exchange proteins and has been employed in numerous studies of the SLC26A family of anion transporters. Presently, the SLC26A6 exchanger is considered to be more sensitive to DIDS than SLC26A3 (DRA) (17, 18), but, with the exception of one study (6), complete dose-response relationships have not been reported prior to the present study. We found that mucosal DIDS is a strong inhibitor of oxalate influx (measured as $J_{\text{muc}}^{\text{ox}}$) into Caco-2 cells, having an EC$_{50}$ of 5 $\mu$M, with maximum inhibition of SLC26A6 occurring at 100 $\mu$M (Fig. 8A). Remarkably, mucosal DIDS inhibition of oxalate efflux (as measured by $J_{\text{muc}}^{\text{ox}}$) from the cell to the mucosal medium was identical to the influx inhibition profile (Fig. 8B). Again, the EC$_{50}$ of 7 $\mu$M, with maximum inhibition of SLC26A6-mediated efflux observed with 100 $\mu$M mucosal DIDS. That 100 $\mu$M DIDS is maximally effective in reducing SLC26A6-mediated oxalate transport in Caco-2 cells is in accordance with studies on mouse ortholog-mediated oxalate uptake expressed in Xenopus oocytes (7, 14). Chloride influx mediated by SLC26A6 (Fig. 9) was similar to that of oxalate influx, being inhibited by DIDS with an EC$_{50}$ of 8 $\mu$M and maximal inhibition occurring at 100 $\mu$M DIDS, although inhibition of $\text{Cl}^{-}$ transport across the whole epithelium (~60%) was not as complete as seen with oxalate (~75%). This implies that there may be additional transcellular $\text{Cl}^{-}$ flux pathways that are less sensitive to DIDS, possibly SLC26A3 (DRA) or SLC26A2 (DTDST).

**Chloride dependence of PAT-1-mediated oxalate exchange.** Using siRNA knockdown we were also able to establish the mucosal chloride dependence of SLC26A6-mediated oxalate flux in Caco-2 cells (Fig. 9). The chloride dependence of $J_{\text{muc}}^{\text{ox}}$ in Con and A6KD monolayers was strong, exhibiting half-maximum activity at less than 20 mM mucosal chloride. The difference between these two curves represents SLC26A6-mediated oxalate flux, and this component exhibited an EC$_{50}$ of ~19 mM mucosal chloride. Although chloride ion is generally considered an important substrate for SLC26A6 on the basis of its ability to cis-inhibit the flux of other transported anions, there is only one previous assessment of chloride inhibition of oxalate uptake over a range of chloride concentrations (7). In the latter study, mouse and human SLC26A6 expressed in Xenopus oocytes exhibited markedly different degrees of cis-inhibition of oxalate uptake by extracellular $\text{Cl}^{-}$. For example, 3 mM extracellular $\text{Cl}^{-}$ reduced mouse slc26a6-mediated oxalate uptake ~40% but was without effect on oxalate uptake mediated by the human ortholog. Moreover, both mouse slc26a6 and human SLC26A6 exhibited highly cooperative dependence of oxalate efflux rate on extracellular chloride, but the EC$_{50}$ for extracellular $\text{Cl}^{-}$ was 8 mM for mouse slc26a6 and 62 mM for human SLC26A6. We have not performed an analogous experiment (trans-stimulation), but in our study (Fig. 10) SLC26A6-mediated oxalate flux was cis-inhibited by mucosal $\text{Cl}^{-}$ in a hyperbolic manner (no obvious cooperativity), and oxalate uptake rates ($J_{\text{muc}}^{\text{ox}}$) and efflux rates ($J_{\text{muc}}^{\text{ox}}$) were remarkably symmetrical in our hands (Figs. 2, 7, 8, 10, 11). At this time there is no clear explanation for these differences in $\text{Cl}^{-}$ affinity beyond the obvious methodological differences. That is, the present report is based on the properties of constitutively expressed SLC26A6 at 37°C using low oxalate concentrations (<1.5 $\mu$M) compared with properties derived from heterologously expressed SLC26A6 at room temperature using higher oxalate concentrations (7). In this regard, it is of interest to note that mammalian prein (slc26a5) expressed in Xenopus oocytes does not exhibit an anion transport function, yet when expressed in CHO cells, slc26a5-mediated uptake of [14C]oxalate and [14C]formate was demonstrated at levels comparable to that mediated by mouse PAT-1 (4).
Symmetry of SLC26A6-mediated anion transport. Short-circuited Caco-2 monolayers that are bathed by the same, well-stirred buffers on both sides of the epithelium do not support net transport of oxalate, sulfate, or chloride when standard radioisotopic techniques are used. This symmetry of unidirectional anion fluxes indicates that transmembrane gradients of countertransport anion species, which may act as driver ions, do not exist or are too small to support detectable secondary active transport. Imposition of a transepithelial chloride gradient by replacing mucosal chloride with gluconate did generate large asymmetries in the unidirectional oxalate fluxes (Fig. 10), chiefly by increasing the magnitude (Fig. 10) was about half that when mucosal chloride alone was replaced. In the latter conditions the cis-effects are the same, but the possible stimulation of Jox is by a trans-effect is much smaller because intracellular chloride is depleted when Cl−-free buffers are present on both sides. We also observed that Jox in the absence of mucosal Cl− (Per I, Fig. 10A) was ~50% smaller than that measured in symmetrical buffers. This reduction in Jox may be due to the fact that oxalate efflux across the apical membrane driven by Cl− influx would be reduced because of the absence of an inwardly directed Cl− gradient.

In A6KD monolayers, mucosal Cl− replacement produced qualitatively similar effects on JSMS and JSMM that were also DIDS inhibitable (Fig. 10B). In this case, the magnitude of the induced net absorptive flux of oxalate in Per I was ~44% that of Con monolayers, which means that under these conditions SLC26A6 mediates at least 56% of net oxalate absorption, an amount similar to the general contribution of SLC26A6 to total oxalate transport in symmetrical buffers. The cis- and trans-effects that were proposed to account for the increase in Jox in Con cells apply to the A6KD monolayers as well, but the magnitudes are smaller because of reduced SLC26A6 expression. A large net oxalate secretion was observed when serosal chloride was replaced by gluconate in both Con and A6KD cells, although in the latter monolayers Jox was again ~41% of the Con. Notably, the magnitude of the net secretory flux in Con and A6KD monolayers was the same as the net absorptive flux observed with mucosal chloride replacement, and both were fully inhibited by 100 μM mucosal DIDS (Fig. 11). Once more, we would explain the changes in unidirectional oxalate fluxes seen with removal of serosal chloride in the same, but opposite, manner proposed for the changes in oxalate transport occurring with removal of mucosal chloride alone. From these studies it appears that the SLC26A6 exchanger can mediate either net absorption or net secretion of oxalate depending on the orientation of counterion driver gradients. This conclusion contrasts with the finding of asymmetric Cl−-oxalate exchange mediated by SLC26A6 expressed in Xenopus oocytes (6, 7) but is consistent with the behavior of SLC26A6 in Caco-2 cells observed here like the symmetry of mucosal DIDS inhibition of JSMS and Jox and the failure to observe any significant net transport throughout this investigation.

Role of SLC26A6 in intestinal oxalate transport. In PAT-1 knockout mice (KO) we previously observed that mPAT-1 mediates a secretory flux of oxalate across the short-circuited ileum (10). In this animal model mucosal DIDS (200 μM) reduced Jox in wild-type (WT) but not in PAT-1 KO mice, suggesting that mPAT-1, like hPAT-1, is strongly DIDS sensitive. Removal of mucosal Cl− increased JSMS in both WT and KO mice but decreased Jox only in WT, suggesting PAT-1-mediated oxalate efflux is largely driven by Cl− influx in the mouse ileum. On the basis of the present study it appears that hPAT-1 could mediate apical oxalate influx or apical oxalate efflux depending on the magnitude and direction prevailing counterion driver gradients as well as the relative affinities of the transported anions. How these findings relate to the temporal and segmental heterogeneity of vectorial oxalate transport in vivo (11, 12) requires, among other factors, a better understanding of the luminal microenvironment and the prevailing direction of transport (net absorption or net secretion) of the more abundant anions.

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