Kidney and Intestine Transport Defects in Slc26a6 Null Mice

Zhaohui Wang¹, Tong Wang², Snezana Petrovic¹, Biguang Tuo³, Brigitte Riederer³, Sharon Barone¹, John Lorenz⁴, Ursula Seidler³, Peter S. Aronson²,⁵, and Manoocher Soleimani¹,⁶

Departments of ¹Medicine and ⁴Physiology, University of Cincinnati, Cincinnati OH; ²Departments of Physiology and ⁵Medicine, Yale University, New Haven CT; ³University of Hannover, Hannover Germany; and ⁶Veterans Affairs Medical Center at Cincinnati, OH.

Running head: Kidney & intestine transport defects in Slc26a6 ko mice

ZW and TW contributed equally to these studies.

Correspondence: Manoocher Soleimani, M.D., Division of Nephrology and Hypertension, Department of Medicine, University of Cincinnati, 231 Albert Sabin Way, MSB 259G, Cincinnati OH 45267-0585. Phone: 513-558-5463; Fax: 513-558-4309.

E-mail: Manoocher.soleimani@uc.edu.
Abstract.

SLC26A6 (PAT1, CFEX) is an anion exchanger that is expressed on the apical membrane of kidney proximal tubule and small intestine. Modes of transport mediated by SLC26A6 include Cl-formate exchange, Cl-HCO\textsuperscript{3-} exchange and Cl-oxalate exchange. To study its role in kidney and intestine physiology, gene targeting was used to prepare mice lacking Slc26a6. Homozygous mutant Slc26a6(-/-) mice appeared healthy and exhibited normal blood pressure, kidney function and plasma electrolyte profile. In proximal tubules microperfused with a low bicarbonate-high chloride solution, the baseline rate of fluid absorption (J\textsubscript{v}), an index of NaCl transport under these conditions, was the same in wild-type and null mice. However, the stimulation of J\textsubscript{v} by oxalate observed in wild-type mice was completely abolished in Slc26a6 null mice (p < 0.05). Formate stimulation of J\textsubscript{v} was partially reduced in null mice but the difference from the response in wild-type mice did not reach statistical significance. Apical membrane chloride-base exchange activity, assayed with the pH sensitive dye BCPCF in microperfused proximal tubules, was decreased by 58% in Slc26a6-/- animals (p<0.001 vs. wild-types). In the duodenum, the baseline rate of bicarbonate secretion, measured in mucosal tissue mounted in Ussing chambers, was decreased by ~30% (p<0.03), whereas the forskolin-stimulated component of bicarbonate secretion was the same in wild-type and Slc26a6-/- mice. We conclude that Slc26a6 mediates oxalate-stimulated NaCl absorption and also contributes to apical membrane Cl-base exchange in the kidney proximal tubule; it also plays an important role in bicarbonate secretion in the duodenum.

Keywords: Chloride absorption, bicarbonate secretion, proximal tubule, duodenum, apical anion exchange.
Introduction.

SLC26A6 (PAT1, CFEX) is a member of a large, conserved family of anion exchangers (SLC26) that encompasses at least ten distinct genes (8, 13, 15, 16, 19, 21, 22, 25, 31, 33, 35, 36, 40, 42). All, excepting SLC26A5 (prestin), function as anion exchangers with versatility with respect to transported anions (8, 13, 15, 16, 19, 21, 22, 25, 31, 33, 35, 36, 40, 42). SLC26A6 was cloned based on homology to DRA (SLC26A3) and pendrin (SLC26A4) (19, 21). In humans, SLC26A6 maps to chromosome 3 and encodes a 738-amino acid protein (21). Immunohistochemical studies in human pancreas localized SLC26A6 to the apical membranes of the duct cells (21). In addition to pancreas, SLC26a6 is expressed on the apical membrane of kidney proximal tubule (19) and villi of the duodenum (40). Functional studies in *in vitro* expression systems demonstrate that SLC26A6 can mediate multiple anion exchange modes including Cl⁻/HCO₃⁻, Cl⁻/oxalate, Cl⁻/hydroxyl and Cl⁻/formate exchanges (18, 19, 21, 40, 41). Similar anion exchange activities had been previously described in apical membranes of kidney proximal tubule and small intestine (3, 4, 28). Based on its immunolocalization in the kidney, and its ability to function in multiple chloride/anion exchange modes, it was postulated that SLC26A6 is a major contributor to NaCl absorption in the proximal tubule (18, 19, 28). In the duodenum, the principal form of apical chloride/base exchange activity is Cl⁻/HCO₃⁻ exchange, which is responsible for bicarbonate secretion in exchange for chloride absorption (1, 9, 17, 30).

To study its role in kidney and intestine physiology, targeted gene disruption was employed to prepare mice lacking Slc26a6. The Slc26a6 null mice appear normal, with normal growth, blood pressure and serum electrolyte profile. Studies in microperfused kidney proximal tubule demonstrate that Slc26a6 null mice have major defects in apical Cl-base exchange and oxalate
stimulated NaCl absorption. In the duodenum, Slc26a6 null mice display significant reduction in bicarbonate secretion, a pathway essential to protection against acid injury.
Experimental Procedures

Construction of the targeting vector and generation of Slc26a6(-/-) mouse. To generate the targeting construct, slc26a6 genomic clones were isolated from a strain of 129/SvJ mouse phage library and partially characterized by restriction endonuclease mapping, DNA sequencing and polymerase chain reaction analysis. A PGK neo cassette was used as vector. The targeting vector was constructed by site-specific mutagenesis. The short arm is 1.2kb long, starting at 21bp downstream of Exon 3 to the end of the genomic clone. The long arm is 8.7kb long, from 5bp upstream of the start codon (ATG) to upstream of Nsi genomic fragment. In this strategy, Exons 1, 2, and part of Exon 3 were replaced by the Neo gene cassette. Ten micrograms of targeting vector was linearized by NotI and then transfected by electroporation of 129 SvEv iTL1 embryonic stem cells. After selection in G418, surviving colonies were expanded, and PCR analysis was performed to identify clones that had undergone homologous recombination. PCR was done using primer pairs that were designed to identify positive clones. One primer is located outside of the short arm, with a sequence of 5’-TAATGGAAGAGGGTGAACCATCTG-3’. The other primer is located in the 5’-promoter region of the Neo gene cassette and has a sequence of 5’-TGCGAGGCCAGAGGCCACTTGTGTAGC-3’. The PCR fragment in colonies expressing the transgene is expected to be 1.4kb long. Figure 1a is a schematic diagram of the recombinant alleles. More than 400 surviving colonies were screened and six colonies (colonies 154, 233, 236, 323, 326 and 381) showed homologous recombination as determined by positive PCR analysis (Figure 1b). The results were verified by Southern blotting and the sequencing of the PCR fragment.
The ES cells from one of the positive colonies (clone 154) were microinjected into C57BL/6J blastocysts and implanted into pseudopregnant female mice. The chimeric mice were generated and following crossbreeding with wild-type C57BL mice, heterozygote animals were bred.

**Animals.** Animals were euthanized with the use of anesthetics (pentobarbital sodium) according to the institutional guidelines and approved protocols.

**RNA isolation and Northern blot hybridization.** Total cellular RNA was extracted from various mouse tissues including duodenum and kidney according to established methods, quantitated spectrophotometrically, and stored at -80°C. Total RNA samples (30 µg/lane) were fractionated on a 1.2% agarose-formaldehyde gel, transferred to Magna NT nylon membranes, cross-linked by UV light and baked. Hybridization was performed according to established protocols (11). The membranes were washed, blotted dry and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). A 32P-labeled full length Slc26a6 cDNA fragment was used for northern hybridizations.

**Immunofluorescence labeling of Slc26a6 in mouse kidney and duodenum.** Slc26a6+/+ and Slc26a6-/- mice were euthanized with sodium pentobarbital overdose and perfused through the left ventricle with 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Duodena were removed, cut in tissue blocks, and fixed in formaldehyde solution overnight at 4°C. The tissues were frozen on dry ice, and 6 µm sections were cut with a cryostat and stored at -80°C until used. Single immunofluorescence labeling was performed as described using Slc26a6 specific antibodies (27) and either Alexa Fluor 488 (green) or Alexa Fluors 568 (red) goat anti rabbit secondary antibodies (27).

**Renal hemodynamic measurements.** Baseline renal function was determined in mice maintained on normal chow. Mice were anesthetized with ketamine (50 µg/g body wt) and
inactin (100 µg/g body weight) and surgically instrumented for renal and blood pressure measurements with catheters placed in the left femoral artery and vein and in the bladder, as described previously (23, 24). Immediately following surgery, a 3 µl/g body wt bolus of 1% FITC-inulin and 3% para-aminohippuric acid sodium salt (PAH) in isotonic saline was administered. This was followed by a maintenance infusion of the same solution at 0.15 µl/min/g body weight. After a 30 minute equilibration period, baseline renal function was determined through two-30 minute urine samples collected through a catheter in the bladder. At the midpoint of each baseline collection, an arterial blood sample (60 µl) was obtained for determination of plasma FITC-inulin and PAH concentrations, and donor blood was administered to replace the lost volume after each sample was obtained. At the end of the second baseline collection, another blood sample was acquired and plasma electrolyte levels were measured using a pH/Blood Gas Analyzer (Bayer, Medfield, MA). Urinary Na and K concentrations were determined using a Corning 480 Flame Photometer (Bayer). GFR was calculated from inulin clearance, and effective renal plasma flow (ERPF) was calculated from PAH clearance. Blood pressure was monitored throughout using a fixed-dome transducer and a PowerLab data acquisition system (AD Instruments, Colorado Springs, CO).

In situ microperfusion in kidney proximal tubule. In situ microperfusion of Slc26a6+/+ and Slc26a6-/- kidney proximal tubules were performed according to established protocols and as described before (39). Age matched Slc26a6+/+ and Slc26a6-/- mice, weighing 28.1 ± 1.14g (+/+) and 26.55 ± 1.71g (-/-), were anesthetized by intraperitoneal injection of Inactin (100mg/kg). After surgical preparation, 0.9% saline was infused into the left jugular vein at a rate of 0.15 ml/h. In brief, proximal convoluted tubules were perfused at a rate of 15 nl/min with a solution containing (in mM): sodium chloride 140, sodium bicarbonate 5.0, potassium chloride
4.0, calcium chloride 2.0, magnesium sulfate 1.0, dibasic sodium phosphate 1.0, monobasic sodium phosphate 1.0, pH 6.7. For measuring volume absorption, 20 µCi/ml of low-sodium \[^3\text{H}\]-methoxy-inulin was added to the perfusion solution. One collection was made in each perfused tubule and two to four collections were taken in the experimental kidney of each animal. The perfused segments were marked with Sudan Black heavy mineral oil, and their lengths determined following dissection of silicone rubber casts. Calculation of the rate of net fluid absorption was based on changes in the concentrations of \[^3\text{H}\]-inulin, and the rates are expressed per mm tubule length. Data are presented as means ± SE. In Table 2, experimental groups were compared with a control group in either the wild-type or null mice by use of Dunnett's test. In Figure 3, all six groups were compared by analysis of variance. Differences were considered significant if P < 0.05.

**Proximal tubule isolation, in vitro microperfusion and apical Cl\(^-\)/HCO\(_3^-\) exchanger activity measurement.** Isolation and microperfusion of proximal tubules in Slc26a6\(^{+/+}\) mice and Slc26a6\(^{-/-}\) mice performed according to established protocols and as described (2, 6, 10, 28). Intracellular pH was measured using 2',7'-bis-(3-carboxypropyl) -5-(and-6)-carboxyfluorescein, acetoxyethyl ester (BCPCF-AM), as described before (2, 6, 10, 28). Excitation wavelengths were recorded at 488 and 440 nm and emission was measured at 520 nm. Digitized images were analyzed by using the Attograph software. Intracellular calibration was performed at the end of each experiment by using high K\(^+\)-nigerin method (10, 28). The apical Cl\(^-\)/HCO\(_3^-\) exchanger was assayed as the rate of intracellular pH acidification (d\(\text{pH}_i\)/dt) upon switching the luminal perfusate from a chloride free to chloride containing solution (28). DIDS, at 300 µM, was present in bath and EIPA, at 300 µM, in the perfusate to block the basolateral NBC1 and apical NHE3, respectively. One tubule per each animal was used.
**Ussing chamber experiments in duodenum.** To examine bicarbonate secretion in the duodenum, mice were anesthetized with 100% CO2 and killed by cervical dislocation. The proximal duodenum was removed, opened along the mesenteric border and stripped of external serosal and muscle layers in Ringer solution with indomethacin (4°C), and mounted in Ussing chambers (window area, 0.65 cm²). Experiments were performed under continuous short-circuited conditions. The mucosal solution contained the following (in mmol/L): Na+ 140, K+ 5.4, Ca²⁺ 1.2, Mg²⁺ 1.2, Cl⁻ 120, gluconate 25, and mannitol 10. The serosal solution contained (in mmol/L): Na⁺ 140, K⁺ 5.4, Ca²⁺ 1.2, Mg²⁺ 1.2, Cl⁻ 120, HCO₃⁻ 25, HPO₄²⁻ 2.4, H₂PO₄⁻ 2.4, glucose 10, and indomethacin 0.001. The osmolalities for both solutions were ~310 mOsm/kg. Bicarbonate secretion was determined under the automatic control of a pH-stat system (PHM290, pH-Stat Controller, Radiometer Copenhagen) (14). After a 30-minute measurement of basal parameters, Forskolin 10µM were added to the serosal side of tissue in Ussing chambers for 40 minutes to measure the changes in duodenal bicarbonate secretion and Isc. Transepithelial short-circuit current (Isc; reported as µeq/cm² h) was measured via an automatic voltage clamp (Voltage-Current Clamp, EVC-4000; World Precision Instruments, Berlin, Germany) and calomel electrodes connected to the chamber baths with 4% agar-3 M KCl bridges.

**³⁶Cl transport measurement.** The 30 second uptake of ³⁶Cl by luminal membrane vesicle suspensions from duodenum, prepared according to established methods (9), was assayed at room temperature in triplicate by rapid filtration technique. The reaction was stopped by ice cold medium. The radioactivity in each filter was assayed by scintillation spectroscopy. Vesicles and all experimental media were continuously gassed with 100% N₂ or 5% CO₂, 95% N₂. The uptake of ³⁶Cl was measured under three different conditions: no pH gradient (pHᵢ/pHₒ 7.5/7.5
without CO₂/HCO₃⁻), outward pH gradient (pHᵢ/pHₒ 7.5/6.0 without CO₂/HCO₃⁻), and outward pH and bicarbonate gradient (pHᵢ/pHₒ 7.5/6.0 with CO₂/HCO₃⁻). The bicarbonate concentration was 25 mM at pH 7.5 and 0 mM at pH 6.0.

**Materials.** ³²P-dCTP and ³⁶Cl were purchased from New England Nuclear (Boston, MA). Nitrocellulose filters and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). RadPrime DNA labeling kit was purchased from Gibco BRL, USA. BCECF was from Molecular Probes, Inc. (Eugene, OR). mMESSAGE mMACHINE™ Kit was purchased from Ambion (Austin, TX).

**Statistical Analyses.** Values are expressed as mean ± SEM. Statistical analysis was examined using Student t-test or ANOVA. P<0.05 was considered statistically significant.
Results

Germline transmission of the disrupted Slc26a6 gene was accomplished by crossing heterozygote mice. In Figure 1c (left and right panels), mRNA expression of Slc26a6 is compared in the duodenum and kidney of wild-type, heterozygote and knockout Slc26a6 mice. Figure 1d (top panels; left and right figures) depicts immunocytochemical labeling of Slc26a6 in the duodenum of wild-type and knockout mice and Figure 1d (bottom panel; left and right lanes) is an Slc26a6 immunoblotting in microsomal membranes isolated from kidneys of wild-type and Slc26a6 null mice. As indicated, there is complete absence of Slc26a6 mRNA and protein in the duodenum and kidneys of KO animals.

Table 1 summarizes the blood pressure, serum electrolyte profile, and parameters of kidney function such as GFR, urine osmolality, urine volume, and urine sodium, potassium and chloride in 5 wild-type and 5 Slc26a6 null mice. As demonstrated in the table, Slc26a6 null animals have normal blood pressure and kidney function as measured by GFR. Urine sodium was similar, while urine chloride showed a trend toward being increased in Slc26a6 null mice.

To determine the contribution of Slc26a6 to apical Cl-/base exchange, isolated proximal tubules (Methods) were perfused with a chloride-free, sodium and bicarbonate containing solutions (sodium 140, chloride 0, gluconate 115, bicarbonate 25, pH 7.4) and intracellular pH was measured using ratiometric imaging and the pH sensitive dye BCPCF (Methods). Apical chloride-base exchange activity was assayed as the initial rate of pH acidification in response to the luminal addition of Cl⁻ in the presence of CO₂/bicarbonate using the following solution (in mM): sodium 140, chloride 115, bicarbonate 25, pH 7.4. Representative tracings (Figure 2a) and results summation (Figure 2b) are included. The rate of apical Cl/HCO₃⁻ exchanger activity was 0.76 +/- 0.05 pH units/min in wild-type animals (n=21 cells in six tubules) and decreased to
0.33 +/- 0.04 pH units/min in knockout animals (n=43 cells in six tubules), a reduction of 58% (p<0.001). The magnitude of intracellular acidification caused by apical Cl/HCO₃⁻ exchanger was 0.51 +/- 0.02 pH units in wild-type animals and decreased to 0.28 +/- 0.02 pH units in knockout animals (p<0.001). The pHᵢ returned to baseline upon switching back to the Cl-free perfusate (Figure 2a). The initial baseline intracellular pH was 7.38 +/- 0.02 in Slc26a6 null mice, a value significantly lower than 7.49 +/- 0.01 in wild-type animals (p<0.001).

To assess the possible role of Slc26a6 in mediating NaCl absorption in the proximal tubule, tubules were microperfused in situ with a late proximal tubule fluid (chloride 140, bicarbonate 5.0, pH 6.7) and Jᵥ measured as an index of NaCl absorption. The results are shown in Table 2 and Figure 3. The baseline rates of Jᵥ measured in the absence of added formate or oxalate were essentially identical in wild-type and null mice (1.51±0.075 and 1.45±0.22 nl/min/mm, respectively). These results are consistent with previous findings that under the above experimental conditions DIDS-sensitive Cl-base exchange does not contribute to NaCl absorption in surface proximal tubules in the absence of added formate or oxalate (37).

Also shown in Table 2 and Figure 3 is that addition of 1 µM oxalate or 50 µM formate to the luminal perfusion solution markedly and significantly stimulates Jᵥ (from 1.51±0.075 nl/min/mm to 2.15±0.18 and 2.19±0.17 nl/min/mm, respectively) in wild-type mice, consistent with previous results in rat and mouse proximal tubule (38, 39). In contrast, stimulation of Jᵥ by oxalate was completely abolished in Slc26a6 null mice (1.42±0.13 nl/min/mm). This finding demonstrates that the presence of functional Slc26a6 is essential for oxalate stimulation of Jᵥ, consistent with the proposed role of apical membrane Cl-oxalate exchange in this process (3, 4).

Unfortunately, the findings concerning the role of Slc26a6 in mediating formate stimulation of Jᵥ were ambiguous. The value for Jᵥ measured in the presence of formate in null mice
(1.70±0.18 nl/min/mm) was intermediate between the baseline rate and the stimulated rate observed in wild-type mice, but was not significantly different from either value. These findings suggest a partial defect in formate stimulated Jv but such a defect is not conclusively proved.

To ascertain the contribution of Slc26a6 in bicarbonate secretion in the duodenum, mucosal duodenal tissue was mounted in Ussing chambers and bicarbonate secretion was assayed using the pH-stat technique (Methods). The results (Figure 4a) demonstrated that at basal state, bicarbonate secretion was decreased by 30.5% (p<0.03), but forskolin-stimulated bicarbonate secretion remained unchanged in Slc26a6-/- mice. Interestingly, the duodenal short circuit current (Isc), measured in Ussing chambers, was the same in both wild-type and ko animals at basal and stimulated states (Figure 4b). Forskolin-induced Isc increase in the murine duodenum strongly correlates with CFTR activation (12, 32). Taken together, these studies demonstrate significant reduction in basal bicarbonate secretion in the duodena of ko animals, which clearly supports the notion that Slc26a6 is a major apical Cl−/HCO3− exchanger in the duodenum. To verify the contribution of Slc26a6 to apical Cl−/HCO3− exchanger activity, the 30 second influx of 36Cl into luminal membrane vesicles isolated from the duodenum was assayed in the presence or absence of an outward pH and bicarbonate gradient (pHi/pHo= 7.5/7.5 vs. 7.5/6.0 + CO2/HCO3−, see Methods). As demonstrated in Figure 4c, in the presence of an outward pH and bicarbonate gradient, the influx of radiolabeled chloride decreased by ~39% in the apical membrane vesicles from duodenum of Slc26a6 null mice (p<0.05, n=3 for each group). The mRNA expression of Slc26a3 (dra) in the small intestine remained unchanged in Slc26a6 null mice (Fig. 4d).
Discussion

The majority of filtered Na\textsuperscript{+}, Cl\textsuperscript{-}, HCO\textsubscript{3}\textsuperscript{-}, and water are reabsorbed in the proximal tubule (3, 4, 5, 7, 26). Studies performed using membrane vesicles and perfused tubules have led to the concept that a major fraction of the filtered Cl\textsuperscript{-} is reabsorbed via different mechanisms of Cl\textsuperscript{-}/base exchange. These mechanisms include Cl\textsuperscript{-}/formate exchange operating in parallel with Na\textsuperscript{+}/H\textsuperscript{+} exchange and H\textsuperscript{+}/formate cotransport, and Cl\textsuperscript{-}/oxalate exchange operating in parallel with SO\textsubscript{4}\textsuperscript{2-}/oxalate exchange and Na\textsuperscript{+}/SO\textsubscript{4}\textsuperscript{2-} cotransport (3, 4). Formate stimulation of apical Na\textsuperscript{+}/H\textsuperscript{+} exchanger NHE3 is an additional and/or alternative mechanism to explain formate stimulation of NaCl absorption in the proximal tubule (29). In addition to Cl\textsuperscript{-}/formate exchange and Cl\textsuperscript{-}/oxalate exchange, Cl\textsuperscript{-}/OH\textsuperscript{-}(HCO\textsubscript{3}\textsuperscript{-}) exchange has also been described as a mode of apical membrane Cl\textsuperscript{-}/base exchange in the proximal tubule (12, 28). Functional expression studies have indicated that Slc26a6 has the ability to operate in all of these exchange modes (18, 19, 21, 40, 41).

The generation of Slc26a6 null mice now allows the determination of which of these exchange modes are actually mediated by Slc26a6 in the proximal tubule \textit{in vivo}, and the extent to which they contribute to transtubular NaCl transport. Our current studies (\textbf{Figure 2}) demonstrate that Slc26a6 is the major apical Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchanger in the proximal tubule, as shown by ~60% reduction in Cl-dependent HCO\textsubscript{3}\textsuperscript{-} transport as detected by intracellular pH measurements in microperfused tubules in Slc26a6 null animals. The residual Cl-dependent HCO\textsubscript{3}\textsuperscript{-} transport in Slc26a6 null mice indicates the presence of additional unidentified anion exchanger(s). Interestingly, the baseline rate of J\textsubscript{v} measured in the presence of a high chloride-low bicarbonate perfusate was essentially identical in wild-type and null mice, suggesting that the apical membrane Cl\textsuperscript{-}/HCO\textsubscript{3}\textsuperscript{-} exchange mediated by Slc26a6 does not contribute to
transtubular NaCl absorption. This conclusion is consistent with the previous observation that the baseline rate of Jv in the proximal tubule is not sensitive to concentrations of DIDS that abolish the increments in Jv induced by formate and oxalate (38).

In striking contrast to the normal baseline rate of Jv in Slc26a6 null mice, the increment in Jv induced by oxalate was completely abolished. This finding is consistent with the concept that SLC26A6 represents the Cl-oxalate exchanger proposed to mediate NaCl absorption by operating in parallel with SO$_4^{2-}$/oxalate exchange and Na$^{+}$/SO$_4^{2-}$ cotransport (3, 4). The increment in Jv induced by formate was blunted in Slc26a6 mice, suggesting a role for Slc26a6 either in mediating Cl-formate exchange coupled to NHE3 and/or in mediating formate entry into the cell to stimulate NHE3. But since the intermediate value for formate-stimulated Jv in null mice was not statistically different from either the baseline or fully stimulated values, no firm conclusion can be drawn concerning this possible role of Slc26a6.

Slc26a6 shows abundant expression in apical membrane of villi of duodenum, where it is presumed to mediate apical Cl$^{-}$/HCO$_3^{-}$ exchange (40), the main mechanism for basal HCO$_3^{-}$ secretion in the small intestine (12, 17, 30, 32). In addition to bicarbonate secretion, the apical Cl$^{-}$/HCO$_3^{-}$ exchanger functions in parallel with the Na$^{+}$/H$^+$ exchanger NHE3 and, as a result, is essential for the electroneutral absorption of Na$^+$ and Cl$^-$ (9, 17, 30). Slc26a6 expression is very low in the colon but high in small intestine (40), a pattern opposite that of DRA (Slc26a3), which is predominantly expressed in colon but is moderately expressed in small intestine (25). Our experiments demonstrate significant reduction in basal bicarbonate secretion in the duodenum in Slc26a6 null mice. There was however a significant component of basal bicarbonate secretion, which remained intact in the duodenum of Slc26a6 null mice, suggesting the contribution of other apical anion exchangers such as Slc26a3 (DRA) to this process. The Cl$^{-}$
Cl/HCO$_3$- exchanger was examined more directly in luminal membrane vesicles isolated from duodenum using $^{36}$Cl influx method (**Figure 4c**). The results demonstrated significant reduction in Cl/HCO$_3$- exchanger activity, confirming the bicarbonate secretion defect that is observed in the duodenum of Slc26a6 null mice (**Figure 4a and b**). The mRNA expression of DRA (Slc26a3) in the small intestine did not change and remained undetectable in the kidney (**Fig. 4d**) suggesting the absence of compensatory upregulation of Slc26a3 in response to Slc26a6 gene deletion in the duodenum and kidney.

In addition to the Cl/HCO$_3$- exchanger, several studies indicate the presence of a HCO$_3$- conductive pathway that mediates bicarbonate secretion into the duodenum (12, 32). Whether the bicarbonate conductive pathway is the same as CFTR or is a distinct anion channel that is regulated by CFTR remains to be resolved. The HCO$_3$- conductive pathway is stimulated by cAMP generation as demonstrated by Isc activation and is not affected by luminal chloride removal (34). Our studies demonstrated that forskolin-stimulated bicarbonate secretion in the duodenum was comparable in wild-type and Slc26a6 null mice (**Figure 4a**). A recent study demonstrated that Slc26a6 stimulation markedly activated CFTR by increasing its overall open probability when both were co-expressed in cultured cells (20). Our current results demonstrated that forskolin-stimulated Isc activation remained the same in wild-type and Slc26a6 null mice, strongly indicating that Slc26a6 does not play an important role in forskolin-activated CFTR activation in murine duodenum. Taken together, our results demonstrate that Slc26a6 plays an important role in basal bicarbonate secretion, but does not contribute to the forskolin-stimulated bicarbonate secretion. Morphometric analysis of kidney structure did not reveal significant abnormalities in epithelial cells of kidney and duodenum in Slc26a6 null animals.
The effects of Slc26a6 deletion on intestine and tubular function are not profound at steady state, with blood pressure, plasma electrolytes and kidney function remaining comparable to wild type animals. It is worth mentioning that several apical chloride absorbing transporters are present downstream from Slc26a6 in the kidney and intestine (with the furosemide sensitive Na-K-2Cl cotransporter in the thick ascending limb, the thiazide sensitive Na-Cl cotransporter in the distal convoluted tubule, pendrin in the cortical collecting duct, and Slc26a3 in the large intestine). It is plausible that one or more of these transporters might show compensatory upregulation in response to Slc26a6 gene deletion, thus blunting the impact of its deficiency on NaCl wasting. Further, it is possible that deficiency of Slc26a6 may give rise to a more significant phenotype in pathophysiologic states such as volume depletion or sodium or chloride depletion in which proximal tubule NaCl reabsorption plays a more essential role than under baseline conditions.

In conclusion, our studies in Slc26a6 null mice demonstrate that Slc26a6 is a major contributor to apical membrane Cl-base exchange and oxalate stimulated NaCl absorption in the kidney proximal tubule and also plays an important role in bicarbonate secretion in the duodenum.
Acknowledgments

These studies were supported by the National Institute of Health Grants DK 62829 (MS), DK 33793 and DK 17433 (PSA), and DK 62289 (TW), grants Se 460/13-1/2 and Se 460/9-4/5 from the Deutsche Forschungsgemeinschaft (US), a National Kidney Foundation grant (SP), and grants from the Department of Veterans Affairs (Merit Review Award) and Cystic Fibrosis Foundation (to M.S). The contribution of Dr. Elizabeth Mann is greatly appreciated. The authors acknowledge the technical assistance of Terry Fettig.
Reference List


25. **Melvin JE, Park K, Richardson L, Schultheis PJ and Shull GE.** Mouse down-regulated in adenoma (DRA) is an intestinal Cl(-)/HCO(3)(-) exchanger and is up-regulated in colon of mice lacking the NHE3 Na(+)/H(+) exchanger. *J Biol Chem* 274: 22855-22861, 1999.


Figure legends:

Fig. 1.

a. **Schematic diagram of the recombinant alleles.** To generate the targeting construct, Slc26a6 genomic clones were isolated from a strain of 129/SvJ mouse phage library and partially characterized by restriction endonuclease mapping, DNA sequencing and polymerase chain reaction analysis. A PGK neo cassette was used as vector. The targeting vector was constructed by site-specific mutagenesis. The short arm is 1.2kb long, starting at 21bp downstream of Exon 3 to the end of the genomic clone. The long arm is 8.7kb long, from 5bp upstream of the start codon (ATG) to upstream of Nsi genomic fragment. In this strategy, Exons 1, 2, and part of Exon 3 were replaced by the Neo gene cassette.

b. **PCR analysis of DNA isolated from surviving ES cells that were electroporated with the targeting construct.** At least six colonies (out of 300) showed homologous recombination as determined by positive PCR analysis. Southern blotting confirmed the results. The identity of the 1.4 kb fragment was verified by sequencing.

c. **mRNA expression of Slc26a6 in duodena and kidneys of wild-type (+/+), heterozygote (+/-) and Slc26a6 null mice (-/-).**

d. **Immunofluorescence labeling (top panel) and immunoblotting (bottom panel) of Slc26a6 in the duodenum and kidneys of wild-type and Slc26a6 null mice.** No labeling was detected in either the duodenum or kidneys of Slc26a6 null mice.
Fig. 2.

Apical chloride/base (Cl⁻/HCO₃⁻) exchanger activity in isolated microperfused tubules of wild-type and Slc26a6 null mice.

a. Representative tracings in wild-type and null animals demonstrating Cl⁻/HCO₃⁻ exchanger activity. DIDS, at 300 µM, was present in bath and EIPA, at 300 µM, in the perfusate to block the basolateral NBC1 and apical NHE3, respectively. One tubule per each animal was used.

b. Results summation. The rate (top panel) and magnitude (bottom panel) of acidification in response to Cl⁻/HCO₃⁻ exchanger activation were decreased significantly in null mice.

Fig. 3.

Oxalate and formate stimulated volume reabsorption (Jv) in proximal tubules of wild-type and Slc26a6 null mice. Effects of oxalate and formate were examined by microperfusion of proximal convoluted tubule *in vivo* in the absence and presence of 1 M oxalate or 50 M of formate in luminal perfusates. Analysis of variance indicated that the values for Jv in the presence of oxalate were different (p < 0.05) between wild-type and null mice.

Fig. 4.

Bicarbonate secretion and Isc measurement in the duodenum of wild-type and Slc26a6 null mice.

a. Bicarbonate secretion at basal state decreased significantly in Slc26a6 null mice but the magnitude of bicarbonate stimulation by cAMP remained comparable in both groups.
b. Short circuit current measurement. \( \text{Isc} \) remained comparable in wild-type and Slc26a6 null mice at basal state and in response to cAMP stimulation.

c. \(^{36}\text{Cl} \) influx in brush border membrane vesicles from duodenum. The 30 second uptake of tracer \(^{36}\text{Cl} \) was measured according to methods and established techniques (ref. 9). The results were normalized per mg membrane protein and are expressed as \% of control (no pH gradient in wild-type animals). As indicated, outward pH and HCO\(_3\) dependent \(^{36}\text{Cl} \) influx (mediated via Cl\(^{-}/\)HCO\(_3\) \(^{-}\) exchange) is decreased significantly (p<0.05, n=3 for each group) in luminal membrane vesicles from duodenum of Slc26a6 null mice.

d. mRNA expression of Slc26a3 (dra) in small intestine and kidneys of wild-type (+/+), heterozygote (+/-) and Slc26a6 null mice (-/-). As indicated, the expression of dra remained unchanged in the small intestine (left panel), indicating the lack of compensatory upregulation of Slc26a3 in response to Slc26a6 deletion. The expression of dra remained undetectable in the kidney (right panel), consistent with published reports indicating the absence of this exchanger in kidney nephrons.
Fig. 1.

a. Schematic diagram of the recombinant alleles.

Targeting Construct of Mouse Slc26a6 (PAT1; CFEX) Gene

In this strategy, exons 1, 2, and 3 were replaced by the Neo gene cassette.
White bars represent exons and restriction enzyme sites are as indicated.
b. PCR analysis of DNA isolated from surviving ES cells that were electroporated with the targeting construct.

*Arrows point to the expected PCR fragment in transgene-expressing ES cells. Clones 154, 213, 236, 323, 326 and 381 express the recombinant allele.*
c. mRNA expression of Slc26a6 in duodena and kidneys of wild-type (+/+), heterozygote (+/-) and Slc26a6 null mice (-/-). Top panel: short exposure; middle panel: long exposure; bottom panel: 28S rRNA.
e. Immunofluorescence labeling or immunoblotting of Slc26a6 in the duodenum or kidneys of wild-type and Slc26a6 null mice.

Top panel: Immunofluorescence labeling in the duodenum.

Left: wild-type; right: Slc26a6 null mouse
Bottom panel: Immunoblotting of Slc26a6 in the kidney. Right lane: wild-type; left lane: Slc26a6 null mouse.
Apical chloride/base (Cl/HCO₃⁻) exchanger activity in isolated microperfused tubules of wild-type and Slc26a6 null mice.

a. Representative tracings in wild-type (top) and null animals (bottom)
b. Results summation. The rate (top panel) and magnitude of acidification (bottom panel) caused by apical Cl⁻/HCO₃⁻ exchanger activation.
Fig. 3.

Oxalate and formate stimulated volume reabsorption ($J_v$) in proximal tubules of wild type and Slc26a6 null mice.
Fig. 4.
Bicarbonate secretion and Isc measurement in the duodenum of wild type and Slc26a6 null mice.

a. Bicarbonate secretion with and without forskolin

Duodenal bicarbonate secretion ± forskolin in Slc26a6 (PAT1; CFEX) ko mice

**N: /- = 5; +/- = 6**
b. Isc measurement with and without forskolin.

Isc measurement ± forskolin in duodenum of Slc26a6 (PAT1, CFEX) ko mice

N: -/- = 5; +/+ = 6
c. $^{36}$Cl influx in brush border membrane vesicles from duodenum.
d. mRNA expression of Slc26a3 (dra) in small intestine (left panel) and kidneys (right panel) of wild-type (+/+), heterozygote (+/-) and Slc26a6 null mice (-/-).

<table>
<thead>
<tr>
<th></th>
<th>Small intestine</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
<td>+/−</td>
</tr>
<tr>
<td>DRA (Slc26a3)</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
Table 1. Blood pressure, GFR and serum and urine electrolytes in wild-type and Slc26a6 null mice.

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>Knock Out</th>
<th>Wild Type</th>
<th>Knock Out</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Body Weight</td>
<td>24.5</td>
<td>0.82</td>
<td>25.5</td>
<td>1.24</td>
</tr>
<tr>
<td>Kidney Weight</td>
<td>0.292</td>
<td>0.016</td>
<td>0.296</td>
<td>0.015</td>
</tr>
<tr>
<td>BP</td>
<td>79.6</td>
<td>3.7</td>
<td>77.7</td>
<td>3.7</td>
</tr>
<tr>
<td>HR</td>
<td>405</td>
<td>26</td>
<td>397</td>
<td>13</td>
</tr>
<tr>
<td>HCT</td>
<td>0.46</td>
<td>0.01</td>
<td>0.47</td>
<td>0.01</td>
</tr>
<tr>
<td>PK</td>
<td>5.0</td>
<td>0.2</td>
<td>4.9</td>
<td>0.2</td>
</tr>
<tr>
<td>PCl</td>
<td>117.9</td>
<td>1.6</td>
<td>116.7</td>
<td>1.7</td>
</tr>
<tr>
<td>PNa</td>
<td>157.8</td>
<td>1.6</td>
<td>159.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Posm</td>
<td>319.3</td>
<td>2.0</td>
<td>320.6</td>
<td>4.5</td>
</tr>
<tr>
<td>V'urine(ul/min)</td>
<td>2.10</td>
<td>0.32</td>
<td>2.02</td>
<td>0.23</td>
</tr>
<tr>
<td>UNa</td>
<td>273.5</td>
<td>40.3</td>
<td>242.0</td>
<td>34.0</td>
</tr>
<tr>
<td>UK</td>
<td>114.1</td>
<td>26.4</td>
<td>194.5</td>
<td>18.2</td>
</tr>
<tr>
<td>UCl</td>
<td>236.9</td>
<td>18.7</td>
<td>296.1</td>
<td>36.3</td>
</tr>
<tr>
<td>Uosm</td>
<td>1090.4</td>
<td>84.4</td>
<td>1491.0</td>
<td>177.2</td>
</tr>
<tr>
<td>UNaV (uEq/min)</td>
<td>0.573</td>
<td>0.089</td>
<td>0.488</td>
<td>0.052</td>
</tr>
<tr>
<td>UKV (uEq/min)</td>
<td>0.239</td>
<td>0.061</td>
<td>0.391</td>
<td>0.054</td>
</tr>
<tr>
<td>UCIV (uEq/min)</td>
<td>0.495</td>
<td>0.062</td>
<td>0.597</td>
<td>0.051</td>
</tr>
<tr>
<td>UOsmV (uEq/min)</td>
<td>2.351</td>
<td>0.272</td>
<td>2.628</td>
<td>0.314</td>
</tr>
<tr>
<td>GFR (ul/min)</td>
<td>526.3</td>
<td>67.8</td>
<td>601.7</td>
<td>65.5</td>
</tr>
</tbody>
</table>

K: potassium; Na: sodium; Cl: chloride; GFR: glomerular filtration rate; osm: osmolality; BP: blood pressure; HR: heart rate; HCT: hematocrit
Table 2. Effect of formate and oxalate on volume reabsorption (Jv) in proximal tubules of wild-type and Slc26a6 null mice.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>V₀   (nl/min)</th>
<th>Vₐ   (nl/min)</th>
<th>L     (mm)</th>
<th>Jᵥ     (nl/min/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wild-type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>15.19±0.02</td>
<td>12.83±0.18</td>
<td>1.57±0.11</td>
<td>1.51±0.075</td>
</tr>
<tr>
<td>Formate</td>
<td>12</td>
<td>15.08±0.04</td>
<td>12.05±0.56</td>
<td>1.46±0.18</td>
<td>2.15±0.18*</td>
</tr>
<tr>
<td>Oxalate</td>
<td>12</td>
<td>15.07±0.05</td>
<td>10.75±0.41</td>
<td>2.01±0.14</td>
<td>2.19±0.17*</td>
</tr>
<tr>
<td><strong>Knockout</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13</td>
<td>15.07±0.05</td>
<td>12.40±0.38</td>
<td>1.90±0.16</td>
<td>1.45±0.22</td>
</tr>
<tr>
<td>Formate</td>
<td>16</td>
<td>15.00±0.06</td>
<td>12.23±0.36</td>
<td>1.71±0.15</td>
<td>1.70±0.18</td>
</tr>
<tr>
<td>Oxalate</td>
<td>10</td>
<td>15.15±0.02</td>
<td>12.58±0.45</td>
<td>1.85±0.19</td>
<td>1.42±0.13#</td>
</tr>
</tbody>
</table>

N: Number of perfused tubules; V₀: perfusion rates; Vₐ: collection rates; L: Tubular length; Jᵥ: Fluid absorption;
*: Significant difference from control value (P<0.05).
#: Significant difference between wild-type and knockout mice (P<0.05).