SLC26A6 and NaDC-1 Transporters Interact to Regulate Oxalate and Citrate Homeostasis

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ABSTRACT

The combination of hyperoxaluria and hypocitraturia can trigger Ca\(^{2+}\)-oxalate stone formation, even in the absence of hypercalciuria, but the molecular mechanisms that control urinary oxalate and citrate levels are not understood completely. Here, we examined the relationship between the oxalate transporter SLC26A6 and the citrate transporter NaDC-1 in citrate and oxalate homeostasis. Compared with wild-type mice, Slc26a6-null mice exhibited increased renal and intestinal sodium-dependent succinate uptake, as well as urinary hyperoxaluria and hypocitraturia, but no change in urinary pH, indicating enhanced transport activity of NaDC-1. When co-expressed in *Xenopus* oocytes, NaDC-1 enhanced Slc26a6 transport activity. In contrast, Slc26a6 inhibited NaDC-1 transport activity in an activity dependent manner to restricted tubular citrate absorption. Biochemical and physiologic analysis revealed that the STAS domain of Slc26a6 and the first intracellular loop of NaDC-1 mediated both the physical and functional interactions of these transporters. These findings reveal a molecular pathway that senses and tightly regulates oxalate and citrate levels and may control Ca\(^{2+}\)-oxalate stone formation.


Formation of calcareous stones is a major health problem, mainly afflicting the kidney\(^1\) and salivary glands.\(^2\) Most stones are Ca\(^{2+}\)-oxalate, with the minority being Ca\(^{2+}\)-phosphate.\(^1\) Ca\(^{2+}\)-oxalate stone formation can result from hyperoxaluria, hypercalciuria, or reduction in the major urinary Ca\(^{2+}\) buffer citrate.\(^3\) Ca\(^{2+}\)-oxalate stones can form in the absence of hypercalciuria when hyperoxaluria is coupled with hypocitraturia. The anion transporter slc26a6 (National Center for Biotechnology Information [NCBI] accession no. NM_134420) has a major role in controlling systemic oxalate metabolism.\(^4\) Slc26a6 is expressed at high levels in most epithelia, including the proximal intestine, renal proximal tubule, salivary glands, and pancreas.\(^5\) Slc26a6 functions as a 1Cl\(^-\)/2HCO\(_3\)\(^-\) or 1Cl\(^-\)/1oxalate and 1Cl\(^-\)/1formate exchanger.\(^7\) Its pivotal role in oxalate homeostasis was demonstrated in slc26a6\(^-/-\) mice, where the most prominent phenotype is increased serum and urine oxalate that lead to Ca\(^{2+}\)-oxalate kidney stones.\(^8\)

Dietary oxalate is an important source of exogenous oxalate and is absorbed by the intestinal epithelium *via* the paracellular pathway.\(^9\) The liver is the main source of endogenous oxalate; however, under physiologic conditions, a small fraction of the bodily oxalate is derived from hepatic production.\(^10\) Studies using slc26a6\(^-/-\) mice showed that increased serum oxalate is the result of impaired intestinal excretion that, in turn, leads to increased filtered renal oxalate load and formation of Ca\(^{2+}\)-oxalate stones.\(^8\) However, under normal
Table 1. Electrolyte profile of wild-type and slc26a6<sup>−/−</sup> mice urine

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sslc26a6&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Slc26a6&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate (mg/24 hr)</td>
<td>0.45 ± 0.07</td>
<td>0.14 ± 0.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Oxalate (mg/24 hr)</td>
<td>0.06 ± 0.01</td>
<td>0.18 ± 0.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total volume (ml/24 hr)</td>
<td>1.6 ± 0.16</td>
<td>1.6 ± 0.19</td>
<td>1.6 ± 0.04</td>
</tr>
<tr>
<td>pH</td>
<td>6.2 ± 0.07</td>
<td>6.07 ± 0.04</td>
<td>18</td>
</tr>
<tr>
<td>Creatinine (mg/24 hr)</td>
<td>0.74 ± 0.05</td>
<td>0.65 ± 0.06</td>
<td>18</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; (mg/24 hr)</td>
<td>0.12 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>18</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt; (mg/24 hr)</td>
<td>0.36 ± 0.04</td>
<td>0.46 ± 0.04</td>
<td>17</td>
</tr>
<tr>
<td>Cl (mEq/24 hr)</td>
<td>0.26 ± 0.02</td>
<td>0.22 ± 0.02</td>
<td>16</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt; (mEq/24 hr)</td>
<td>0.08 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>18</td>
</tr>
<tr>
<td>SO&lt;sub&gt;4&lt;/sub&gt; (mmol/24 hr)</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>18</td>
</tr>
</tbody>
</table>

Urine samples were collected in 3 consecutive days from six mice from each group and analyzed for the listed electrolytes and metabolites. NS, not significant.

RESULTS

Urine Oxalate, Citrate, pH, and Na<sup>+</sup>-Dependent Dicarboxylic Uptake in slc26a6<sup>−/−</sup> Mice

Knockout of slc26a6 was reported to have increased urinary oxalate, resulting in kidney stones. The slc26a6<sup>−/−</sup> mice developed in our laboratory also have massive formation of Ca<sup>2+</sup>-oxalate stones in the kidney and stone accumulation in the urinary bladder (not shown). It was assumed that the hyperoxaluria was the sole driver for lithogenesis in these animals, but a critical determinant in formation of Ca<sup>2+</sup>-oxalate stone is urinary citrate. To determine the state of citrate in the slc26a6<sup>−/−</sup> mice and monitor the effect of slc26a6 on urinary citrate/oxalate homeostasis, we collected 24-hour urine samples from slc26a6<sup>−/−</sup> and wild-type (WT) mice over 3 consecutive days and performed a kidney stone profile (Table 1). Figure 1, A–C, shows about a four-fold increase in urine oxalate, a prominent three-fold reduction in urinary citrate, and no significant change in urinary pH in slc26a6<sup>−/−</sup> mice. Although the original study analyzing electrolytes in another slc26a6<sup>−/−</sup> mouse line reported no change in urinary citrate, successive analysis with the same mouse line revealed reduction in urinary citrate, as found here. The hypocitraturia cannot be explained by the known causes, including K<sup>+</sup> deficiency, increased acid load, and frank metabolic acidosis. This urine composition greatly increases the Ca<sup>2+</sup>-oxalate stone risk.

Urinary citrate is determined by proximal tubule citrate absorption, which is mediated by Na<sup>+</sup>-dicarboxylate co-transporter NaDC-1. Reduced urinary citrate suggests increased citrate absorption and NaDC-1 activity in the slc26a6<sup>−/−</sup> mice. In preliminary experiments, the signal-to-noise ratio of Na<sup>+</sup>-dependent succinate transport in renal brush-border membranes preparation from mice kidney was too small to resolve the effect of slc26a6 on the transport. Therefore, we prepared kidney cortical slices (see Concise Methods section). Figure 1D shows that deletion of slc26a6 increased the Na<sup>+</sup>-dependent succinate uptake by about 70%. A limitation of the slice preparation is that altered succinate uptake could not be assigned to the luminal membrane. However, because...
slc26a6 is expressed in the luminal membrane, the increase most likely reflects increased activity of NaDC-1. Since slc26a6 and NaDC-1 are expressed at a high level in the jejunum, and slc26a6-mediated jejunal oxalate excretion has a major role in controlling systemic oxalate metabolism, we also used jejuna sheets from slc26a6−/− mice collected during 3 consecutive days. Notice the significant decreased citrate and increased oxalate with minimal change in urinary pH in slc26a6−/− mouse urine. (D) Na+-dependent succinate uptake by kidney slices obtained from wild-type and slc26a6−/− mice.

**Figure 1.** Deletion of slc26a6 in mice upregulates Na+-dependent dicarboxylate uptake. (A–C) Analysis of urine citrate (A), oxalate (B), and pH (C) in wild-type and slc26a6−/− mice collected during 3 consecutive days. Notice the significant decreased citrate and increased oxalate with minimal change in urinary pH in slc26a6−/− mouse urine. (D) Na+-dependent succinate uptake by kidney slices obtained from wild-type and slc26a6−/− mice.

An important question is whether NaDC-1 senses the activity of slc26a6: namely, whether the inhibition is more significant when slc26a6 is active. To address this question we compared the inhibition of NaDC-1 by active slc26a6 and by the inactive slc26a6(E357K) mutant, which lacks all forms of slc26a6 activity. Interestingly, Figure 3 shows that although both active and inactive slc26a6 inhibit NaDC-1, active slc26a6 inhibited NaDC-1 about two-fold more than did inactive slc26a6(E357K). These findings indicate that once interacting, slc26a6 inhibits NaDC-1, but when slc26a6 is active it may resume a conformation that enhances interaction with NaDC-1. This may function as a sensing mechanism whereby inhibition of NaDC-1 becomes more effective when oxalate excretion takes place.

Inhibition of NaDC-1 by slc26a6 raised the question of whether NaDC-1 reciprocally affects the activity of slc26a6. In Figure 4, A and B, we measured the effect of NaDC-1 on slc26a6-mediated Cl−/oxalate exchange in oocytes expressing slc26a6 alone or coexpressing slc26a6 and NaDC-1. NaDC-1 does not transport oxalate (Supplemental Figure 2). Addition of 1 mM oxalate to oocytes bathed in Cl−-free solution resulted in an outward current due to Cl−/oxalate exchange. The slow reduction in the current is due to accumulation of intracellular oxalate, which accounts for the overshoot in the inward current upon readdition of Cl−. Coexpression of NaDC-1 increased Cl−/oxalate exchange by about 30% (Figure 4B). Another important function mediated by slc26a6 is 1Cl−/2HCO3− exchange. Figure 4, C and D, shows that NaDC-1 similarly increased the slc26a6-mediated 1Cl−/2HCO3− exchange without affecting the stoichiometry of the exchange. This may function to increase the pH at the NaDC-1/slc26a6 microdomain to limit access of divalent citrate to NaDC-1. Figure 4E shows that surface expression of slc26a6 is not increased by NaDC-1, indicating that NaDC-1 regulates slc26a6 activity rather than trafficking. The results in Figures 2–4 were obtained with opossum NaDC-1 that was used originally to characterize NaDC-1. Supplemental Figure 4 shows that similar results were obtained with human NaDC-1 (NCBI accession no. BC096277). Taken together, the results in Figures 1–4
indicate that NaDC-1 and slc26a6 interact and reciprocally regulate their activities; NaDC-1 activates slc26a6 while slc26a6 inhibits NaDC-1, and the inhibition is modulated by the activity of slc26a6.

The slc26a6 STAS Domain and NaDC-1 ICL1 Mediate the Reciprocal Regulation

Our next aim was to identify the molecular determinants that mediate the interaction between NaDC-1 and slc26a6. We have previously identified the SLC26 transporter STAS domain as a protein–protein–interacting domain that participates in regulation of other transporters, such as cystic fibrosis transmembrane conductance regulator, and therefore tested whether it may mediate the slc26a6-NaDC1 interaction. Figure 5, A and B, shows that both the slc26a6 and slc26a3 STAS domains inhibit NaDC-1 to the same extent measured with the full-length transporters. In addition, the slc26a6 STAS domain and NaDC-1 can be co-immunoprecipitated (Figure 5C). These observations show that slc26a6 inhibits NaDC-1 via STAS domain-mediated interaction.

To locate the NaDC-1 site that interacts with the STAS domain, we generated the five NaDC-1 fragments illustrated in Figure 6A, based on the 11 transmembrane domain topology model of NaDC-1 (Pajor and Supplemental Figure 5). All fragments except NaDC-1-M45 interacted with slc26a6, suggesting that NaDC-1(45–118) that includes the first NaDC-1 ICL1 is probably the slc26a6 interacting site. To ascertain that the lack of interaction of NaDC-1(1–45) and the interaction of NaDC-1(1–118) was not promoted by the mCherry tag, we prepared hemagglutinin-tagged fragments and obtained the same results (Figure 6B). We also prepared NaDC-1 with truncated ICL1 (NaDC-1[ΔICL1]) (Figure 6C). Unfortunately, this construct did not traffic to the plasma membrane, as evident from the presence of only the nonmature form of the protein (Figure 6C). This precluded determination of the effect of the truncation on NaDC-1 activity and co-regulation by slc26a6. Nevertheless, we used this construct to test for interaction with slc26a6, and as shown in Figure 6D, truncation of ICL1 markedly reduced interaction of NaDC-1 with slc26a6. Together, the results in Figure 6 indicate that NaDC-1 ICL1 mediates the interaction with slc26a6.

To further establish the role of ICL1 in the regulation of slc26a6, we prepared a His-tagged ICL1 construct that includes residues 35–119 of NaDC-1 and tested its interaction with and
activation of slc26a6. Figure 7, A and B, shows that ICL1 activated slc26a6-mediated Cl\(^-\)/oxalate exchange to the same extent as NaDC-1. Furthermore, ICL1 interacts with both full-length slc26a6 and the slc26a6-STAS domain (Figure 7C). Hence, the interaction between NaDC-1 and slc26a6 occurs intracellularly and is mediated by the slc26a6 STAS domain and the NaDC-1 ICL1.

DISCUSSION

We describe a molecular mechanism via interaction of two transporters that can set the oxalate/citrate level in several organs that express the oxalate transporter slc26a6 and the citrate transporter NaDC-1, and that experience variable loads of these molecules. Biochemical and functional analyses reveal interaction between slc26a6 and NaDC-1 mediated by specific modules. The STAS domains of several SLC26 transporters—slc26a3, slc26a6, and SLC26A9—were shown to function as protein-protein–interacting domains to mediate the mutual regulation of the SLC26 transporters and CFTR. The slc26a6 STAS domain also interacts with carbonic anhydrase II. Here we show that the STAS domains of slc26a6 and slc26a3 interact with NaDC-1 and inhibit NaDC-1 activity, similar to the full-length protein. It is of interest that slc26a3 and slc26a6 but not slc26a2 inhibited the activity of NaDC-1.
Supplemental Figure 3). This may relate to the variability in the STAS domain. The highest variability and the most prominent difference between the STAS domains of the SLC26 transporters is at a defined region referred to as the intervening sequence or variable loop found between the first α-helix and third β-sheet.30 This region is predicted to be disordered in solution and contains many positively charged residues. Slc26a2 lacks the intervening sequence loop.

The molecular identity of NaDC-1 has been known for several years.20 Despite the low sequence similarity with mammalian NaDC-1, the bacterial Na+/citrate symporter citS and NaDC-1 share many features. Both proteins are predicted to have 11 transmembrane domain spans, which is supported by experimental data.25,31 This was further established recently by the resolved structure of the bacterial Na+-dicarboxylate transporter VcINDY.32 NaDC-1 and VcINDY also mediate Na+/citrate cotransport. Here we used the 11 transmembrane domain topology of NaDC-1 (Supplemental Figure 5) to identify ICL1 as the NaDC-1 module that interacts with the STAS domain to enhance slc26a6 activity. Significantly, ICL1 is as effective as full-length NaDC-1 in the activation of slc26a6, indicating that STAS-ICL1 domains interaction underlies the molecular mechanism of citrate/oxalate regulation by slc26a6 and NaDC-1.

That ICL1 and the STAS domain recapitulate the interaction and mutual regulation of NaDC-1 and slc26a6 indicates that interaction between the transporters is required for the regulation. Indeed, the mutant slc26a6(E357K) that lacks all slc26a6 functional modes7 inhibited NaDC-1. However, slc26a6(E357K) was less effective than slc26a6 in inhibiting NaDC-1, suggesting two levels of regulation of NaDC-1 by slc26a6. The inhibition is mediated by direct interaction between the transporters and is augmented when slc26a6 is engaged in transport. The requirement for an active slc26a6 may reflect favoring of a STAS domain conformation that interacts better with ICL1, sensitivity of NaDC-1 to pH, the membrane potential, or all of the above. Our findings are not sufficient to firmly support any of these possibilities, although sensitivity of NaDC-1 to pH and the membrane potential is well established (see below). The present findings raise the possibility that physiologically NaDC-1 activity is inhibited mostly when slc26a6 is actively transporting oxalate.

The mutual and reciprocal regulation of slc26a6 and NaDC-1 suggests the sensor-regulatory molecular mechanism for oxalate/citrate homeostasis proposed in Figure 8. Slc26a6-NaDC-1 exists in a complex in which slc26a6 is active to excrete oxalate to the urine, in addition to the amount of oxalate that is already present in the filtrate. Under these conditions, high urine citrate is required to chelate Ca2+ and prevent its binding to oxalate. Inhibition of NaDC-1 by slc26a6 in the complex inhibits citrate absorption to increase luminal citrate, thus providing a safe haven to increase luminal oxalate. The physiologic relevance of the stimulation of slc26a6 activity by NaDC-1 is not known with certainty. However, it is possible that activation of slc26a6 depolarizes the plasma membrane due to Cl-/Ox2- exchange to further reduce the 3Na+/dicarboxylate transport by NaDC-1. In addition, NaDC-1 may be modulated by the other slc26a6 function of Cl-/2HCO3 exchange. This can function as a negative feedback mechanism to limit oxalate uptake by increasing the HCO3 concentration in the microenvironment around the NaDC-1/slc26a6 complex in the tubular lumen and dampening NaDC-1 activity by reduction of substrate availability33 and direct pH gating.34 The result is further reduction in citrate absorption to increase tubular citrate. The hypocitraturia in slc26a6-/- mice cannot be
accounted for by dietary difference in acid load (equivalent urinary sulfate and ammonium excretion) or potassium status (equivalent plasma and urinary potassium), strongly supporting an intrinsic change in proximal tubule citrate reabsorption. The slc26a6-NaDC-1 homeostatic system is likely to operate in several organs and to be disrupted in disease states. In the present studies we showed that deletion of slc26a6 increases intestinal NaDC-1 activity. Stones are very common in salivary glands and may result from malfunctioning or overwhelming of the slc26a6-NaDC-1 homeostatic system. Indeed, recent studies reported Ca2+-oxalate stones in salivary glands of several patients with CKD. Intriguingly, a recent study found a subgroup of stone formers with slight alkalinuria (pH 6) that showed suboptimal citrataturic response to standard alkali therapy. The authors speculated on dysregulation of NaDC-1 as a possible reason for this condition. We show here that slc26a6−/− mice indeed have significantly reduced citrate and high oxalate. Maintenance of inhibition of NaDC-1 by slc26a6 may be an important homeostatic mechanism that is deranged in stone formers. It will be of interest to explore the functioning and the significance of the slc26a6-NaDC-1 homeostatic system in multiple organs.

CONCISE METHODS

Animal Care and Metabolism Experiments
All of the work on mice was conducted following the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center and the National Institute of Craniofacial and Dental Research, National Institutes of Health (NIH). Wild-type and slc26a6−/− mice were individually housed in MMC100 metabolic cages (Hatteras Instruments Inc.). All mice were on rodent diet NIH 31 and tap water during the experiments. After acclimatization, 24-hour urine samples were collected over the course of 3 consecutive days. The collected samples were analyzed for urine electrolytes, and the results are summarized in Supplemental Table 1.

Succinate Uptake
For all experiments, mice were euthanized with CO2.

Kidney Slices
The two kidneys were excised into PBS (pH, 7.4), and the capsules were removed by pinching. Fresh kidneys were embedded in 4% agarose blocks in PBS and polymerized at room temperature. The agarose-kidney block was cooled and 1-mm-thick slices were cut using Vibratome (Electron Microscopy Sciences model OTS-3000) (insert in Supplemental Figure 6A). The kidney slices were separated from the agarose, and the cortical section was cut out and placed in ice chilled PBS. Photo images on top of a graded paper used as scale were taken for determining the kidney cortical area (Supplemental Figure 6A).

Intestinal Slices
The mid-jejunum were removed, and the tissue was transferred immediately into ice-chilled PBS (pH 7.4), cut along the mesenteric border, cleaned, and cut to small pieces of about 5 × 5 mm. The tissues were placed in six-well dishes fully stretched with 27-gauge needles and photo images on top of a graded paper used as scale were taken for determining the epithelium area (Supplemental Figure 6B).

Image analysis of all tissues was performed with ImageJ v1.43u software (NIH). The tissues were then incubated in a water bath and agitated (100 oscillations/min) for 5 minutes at 37°C in 24-well dishes containing 500 μl of incubation solution (in mM): 5 KCl, 10 HEPES, 50 BSA, 100 mg/l streptomycin, 100 mg/l penicillin, and 1× insulin-transferrin-sodium selenite (ITS) on 10% CO2.
10 glucose, 140 NaCl or NMDG-Cl, pH of 7.4. Subsequently, the tissues were transferred to wells containing uptake solution supplemented with 0.1 mM (kidney) or 0.5 mM (intestine) succinic acid and 1 µCi 14C-succinic acid (ViTrax Inc.) per 0.12 µmol (kidney) or 0.7 µmol (intestine) of cold succinate. Tissues were incubated by agitation for an additional 2 minutes (kidney) or 5 minutes (intestine) at 37°C. The samples were then washed in ice-chilled PBS for 2 hours after cRNA injection.

**Preparation and Injection of Oocytes**

Oocytes were obtained by partial ovariectomy of female *Xenopus laevis* (*Xenopus Express*, Beverly Hills, FL), as previously described. Briefly, animals were anesthetized and follicle cells were removed in OR-2 calcium-free medium. Defolliculated oocytes in stages V to VI were collected under a microscope and maintained at 18°C overnight in ND96 solution. Oocytes were injected with cRNA using a Nanoliter 2000 injector (World Precision Instruments Inc.). Oocytes were incubated at 18°C in ND96 solution with pyruvate and antibiotics and were tested 48–96 hours after cRNA injection.

**Voltage, Current, pH, and Cl^- Measurement in oocytes**

Current, voltage, pHo, and [Cl^-], recordings were performed with two-electrode voltage clamp as described elsewhere. In brief, current was recorded using Warner Instrument Corp. amplifier model OC-725C and digitized via an A/D converter (Digidata 1322A; Axon Instruments Inc.). Slc26a6-dependent currents were recorded at a holding membrane potential of 0 mV and NaDC-1-dependent currents at a membrane potential of −30 mV. Data were analyzed using the Clampex 8.1 system (Axon Instruments Inc.). The electrode tips were filled with 0.5 µl of H+ or Cl^- exchanger resin, hydrogen ionophore I, cocktail B (Sigma-Aldrich), and Cl^-sensitive liquid ion exchanger 477913 (Corning), respectively, and then backfilled with a 3 M KCl solution. During measurements, two channels were used for ion-sensitive measurement and another was used to record or control the membrane potential. The pH and Cl^- signals were extracted by subtracting the membrane potential signal from the ion-selective

**Deletion mutations were generated using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent).**

All constructs were verified by sequencing and Western blot analysis of the protein product. The genes cloned into vectors pCDNA 3.1(+), pCMV-SPORT6, pCMV6-AC-mKate, and pCMV-AC-myc-His were linearized using relevant restriction enzymes and transcribed *in vitro* with T7 or sp6 mMessage mMachine or mMessage mMachine ultra kits (Applied Biosystems). To prepare the STAS domains, human slc26a6 (NM_022911) residues 530–742 were amplified by PCR and subsequently cloned into pCMV-HA using XhoI and NotI restriction sites (pCMV-HA-STAS-A6). Mouse slc26a6 (NM_134420) residues 508–718 were similarly cloned into pCMV-Myc vector (pCMV-Myc(STAS-A6). For *Xenopus* oocyte expression, pCMV-HA-STAS-A6 and pCMV-Myc(STAS-A6 inserts were subcloned into pCDNA 3.1(−) using flanking ApaI and NotI restriction sites. pXBG-ev1(STAS-A3 (residues 518–713) was also expressed in *Xenopus* oocytes and the construct is reported by Ko *et al.*

**Plasmid Construction and cRNA Preparation**

In the current study, we used the untagged opossum NaDC-1 (NCBI accession no. AY186579) pCDNA 3.1(+)/opNaDC-1 clone and mouse slc26a6 (NCBI accession no. NM_134420) pCMV-SPORT6/mslc26a6. The open reading frame of opossum NaDC-1 was subcloned into pRK5-HA and p3xFLAG-CMV-7.1/mCherry plasmids. The open reading frame of mouse slc26a6 was subcloned into pEGFP-C1 and pCMV6-AC-mKate plasmids. Human NaDC-1 (NCBI accession no. BC096277) was cloned into pCMV-AC-myc-His. All NaDC-1 site-directed mutants were generated by PCR with QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent).
electrodes signal using Origin software (version 8.1; OriginLab). For simultaneous measurement of pH and [Cl⁻], in oocytes, a three-electrode method was used as described previously.⁶ The following solutions were used as indicated in the figures: Standard HEPES-buffered ND96 oocyte regular medium containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES, pH of 7.5. Cl⁻ free solutions for oocytes were prepared by replacing CaCl₂ with MgSO₄·H₂CO₃ by replacing 25 mM NaCl or Na+-gluconate with 25 mM NaHCO₃. The result is retention of citrate in the filtrate and perhaps other luminal fluids to chelate part of the Ca²⁺ to allow safe oxalate excretion. When minimal oxalate excretion is required, the interaction between slc26a6 and NaDC-1 relaxes to increase the activity of NaDC-1 and citrate absorption and salvage. Disruption of this regulation in disease states leads to Ca²⁺/oxalate stone formation.

**Surface Expression and Co-immunoprecipitation**

Surface expression was analyzed by biotinylation. Because oocytes transport biotin, biotinylation assays are not reliable in oocytes. Thus, biotinylation was performed using HEK cells. HEK cells expressing slc26a6 and/or NaDC-1 were incubated with 0.5 mg/ml EZ-LINK Sulfo-NHS-LC-biotin (Thermo Scientific) for 30 minutes on ice, quenched by incubation with 50 mM glycine for 10 min and washed twice with PBS. Cells were collected by scraping and centrifugation for 20 minutes. Lysates were prepared by incubating the cells in ice-cold lysis buffer containing PBS, 10 mM Na-pyrophosphate, 50 mM NaF, 1 mM Na-orthovanadate, 1% Triton X-100, and protease inhibitors cocktail. Total protein concentration was determined by the Laurie method. Biotinylated proteins were then isolated with avidin beads by overnight incubation with the lysates and recovered by heating in SDS sample buffer at 37°C for 30 minutes. Similar lysis buffer was used for co-immunoprecipitation; extracts were incubated O.N. with the indicated antibodies, and complexes were collected with protein A or G Sepharose beads by incubation for 4 hours at 4°C. Beads were collected by centrifugation and washed three times with lysis buffer; proteins were recovered by heating in SDS sample buffer at 37°C for 30 minutes. The samples were subjected to SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes.

**Statistical Analyses**

All results are given as mean ± SEM, and significance was analyzed by t test or ANOVA using Origin software 8.1.

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**DISCLOSURES**

None.

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