STE20/SPS1-related proline/alanine-rich kinase (SPAK) is critical for sodium reabsorption in isolated, perfused thick ascending limb

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1Department of Medicine, Division of Nephrology, UT Southwestern Medical Center, Dallas, Texas; 2Department of Pediatrics, UT Southwestern Medical Center, Dallas, Texas; and 3Department of Medicine, Division of Nephrology, Tri-Service General Hospital, National Defense Medical Center, Taipei 114, Taiwan

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Cheng C-J, Yoon J, Baum M, Huang C-L. STE20/SPS1-related proline/alanine-rich kinase (SPAK) is critical for sodium reabsorption in isolated, perfused thick ascending limb. Am J Physiol Renal Physiol 308: F437–F443, 2015. First published December 4, 2014; doi:10.1152/ajprenal.00493.2013.—SPAK [STE20 (sterile 20)/SPS1-related proline/alanine-rich kinase] kinase consists of a full-length (FL-) and an alternatively spliced kidney-specific (KS-) isoform. SPAK regulates the NaCl cotransporter (NCC) in the distal convoluted tubule (DCT). The relative abundance and role of FL- vs. KS-SPAK in regulating Na⁺-K⁺-2Cl⁻ cotransporter (NCC2) in thick ascending limb (TAL) are not completely understood. Here, we report that FL-SPAK mRNA was the most abundant in medullary TAL (mTAL), followed by cortical TAL (cTAL) and DCT. KS-SPAK mRNA abundance was relatively lower than FL-SPAK. The ratios of FL-SPAK to KS-SPAK in mTAL, cTAL, and DCT were 12.3, 12.5, and 10.2, respectively. To examine the role of SPAK in the regulation of sodium transport in TAL, we used in vitro microperfusion of mTAL and cTAL isolated from wild-type (WT) and SPAK knockout mice (SPAK-KO) that lack both FL- and KS-SPAK. The rates of sodium absorption in cTAL and mTAL of SPAK-KO mice were 34.5 and 12.5% of WT tubules, respectively. The mRNA levels of related OSR1 kinase and SPAK protease Dnpep in SPAK-KO tubules were not significantly different from WT tubules. We next examined the role of SPAK in the regulation of sodium reabsorption by vasoressin in TAL. Vasopressin increased sodium reabsorption by ~80% in both mTAL and cTAL from WT mice. While baseline sodium reabsorption was lower in SPAK-KO tubules, vasopressin increased sodium reabsorption over twofold. In conclusion, the combined net effect of SPAK isoforms on sodium reabsorption in TAL is stimulatory. SPAK is not essential for vasopressin stimulation of sodium reabsorption in TAL.

SPAK; sodium; thick ascending limb; vasopressin

SPAK [STE20 (sterile 20)/SPS1-related proline/alanine-rich kinase] is a member of germinal center kinase IV subfamily of STE20-related protein kinases that shares 67% amino acid sequence homology with another mammalian STE20 kinase oxidative stress-responsive kinase-1 (OSR1) (25, 26). Both SPAK and OSR1 are enriched in transporting epithelia, especially renal tubules. In vitro studies showed that SPAK and OSR1 enhance the activities of Na⁺, Cl⁻ cotransporter (NCC) and Na⁺, K⁺, 2Cl⁻ cotransporter isofoms 1 and 2 (NCC1/2) via phosphorylating a cluster of threonine and serine residues in the N terminus of these transporters (6, 19, 23). By analysis of proteins that interact with with-no-lysine (WNK) kinases, SPAK and OSR1 were found to be downstream substrates of WNK kinases (14, 27). WNK kinases phosphorylate and activate SPAK and OSR1, which in turn phosphorylate and activate NCC and NKCC1/2. Heterozygous mutations in WNK1 and WNK4 genes cause an autosomal-dominant hypertension known as pseudohypoaldosteronism type II (PHAII) (29). Polymorphisms within introns of the SPAK gene are also associated with hypertension (28). These findings implicate the importance of WNK-SPAK/OSR1 in renal salt reabsorption (3, 13).

To understand the physiological role of WNK-SPAK/OSR1 in the kidney, mouse models with genetic modification of WNK, SPAK, or OSR1 have been created. Yang et al. (31) reported that phosphorylation of SPAK/OSR1 and the abundance and activity NCC/NKCC2 were increased in kidneys of mice carrying a WNK4D561A/+ PHAII disease mutation, supporting that upregulation of WNK-SPAK/OSR1-NCC/NKCC2 signaling cascade is causative for the hypertension in patients with PHAII. Several mouse models have been established to study the role of SPAK in renal Na⁺ transport (12, 21, 30). Rafiqi et al. (21) generated the kinase-deficient SPAK243A knock-in (SPAK243A/243A) mice in which the critical threonine 243 in T-loop for kinase activity was mutated to alanine. Yang et al. (30) generated a SPAK knockout (SPAK-KO) mouse model by targeted deletion of exon 9 and 10 of SPAK gene. Both of these mouse models displayed evidence of decreased NCC activity and salt-wasting. However, the effect of SPAK on NKCC2 was not consistent. While SPAK243A/243A knockin mice had reduced total and phosphorylated levels of NKCC2 (21), SPAK-KO mice exhibited increased phosphorylation and activity of NKCC2 (30).

McCormick et al. (12) also studied the role of SPAK using a mouse model in which the function of SPAK gene is disrupted by a gene trap insertion within the exon 6. This study also reported that a shorter form of SPAK was specifically expressed in the kidney, and named this splice variant kidney-specific SPAK (KS-SPAK). The transcript of KS-SPAK was alternatively initiated from a unique exon 5a and the encoded KS-SPAK protein lacks the kinase domain of the full-length SPAK (FL-SPAK). KS-SPAK was highly expressed in the thick ascending limb (TAL) and is thought to be a functional antagonist of FL-SPAK. Based on the relative preferential expression of KS-SPAK over FL-SPAK in TAL, McCormick et al. proposed that the combined net effect of FL- and KS-SPAK isoforms plays an inhibitory role on NKCC2 in TAL. Recently, it was reported that a protease aspartyl aminopeptidase (Dnpep) was responsible for proteolysis of FL-SPAK and production of smaller SPAK fragments detected in the kidney lysate (11). These smaller SPAK fragments exerted an inhibitory effect on NKCC2 in vitro. It was suggested that
proteolysis by Dnpep may be an alternative mechanism for the production of inactive and potentially dominant-negative SPAK in vivo.

Vasopressin stimulates sodium (Na⁺) reabsorption in the TAL. The role of cyclic AMP-dependent protein kinase A (PKA) signaling pathway in the activation of NKCC2 by vasopressin is well-established (1). Since SPAK is a potent activator of NKCC2, it is possible that SPAK participates in the vasopressin-induced NKCC2 stimulation of Na⁺ reabsorption in TAL. Here, we study the relative abundance and distribution of SPAK isoforms and the related OSR1 and the SPAK protease Dnpep in renal tubules and directly measure the Na⁺ reabsorption rate in SPAK-KO and control TAL using in vitro microperfusion, and the role of SPAK in vasopressin regulation of Na⁺ reabsorption in TAL.

METHODS

Animals. Creation of SPAK-KO mouse and genotyping have been reported previously (30). In these studies, SPAK-KO mice were backcrossed >10 generations to achieve congenic background of C57BL/6. All experimental procedures involving these animals were performed in accordance with relevant laws and institutional guidelines approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center at Dallas and National Defense Medical Center at Taipei, respectively. The experiments were performed on SPAK-KO and wild-type (WT) C57BL/6 mice at 8–10 wk of age. The mice were kept in a 12:12-h day-night cycle and fed a normal rodent chow and plain drinking water ad libitum.

Microdissection and reverse transcription-PCR. Slices of kidney were placed into a prewarmed collagenase type I (Worthington, Lakewood, NJ; 1.5 mg/ml dissolved in DMEM/F12) solution in a 15-ml tube, which was then shaken vigorously on a titer plate shaker at 37°C for 10–15 min. After digestion, the individual nephron segments were dissected in 4°C Hank’s solution and transferred by adhering the tubules to small glass beads (0.5-mm diameter, Thomas Scientific, Swedesboro, NJ) and then transferring the beads to 1.5-ml tubes containing 0.6 ml RNase inhibitor-containing lysis buffer. Total RNA was immediately extracted using Quick-RNA MicroPrep kit (Zymo Research, Irvine, CA). Reverse transcription was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA). Quantitative real-time PCR was carried out on MyiQ single color RT-PCR detection system (Bio-Rad, Hercules, CA). We verified that no amplification was produced when reverse transcription was omitted from the sample. Sequences of primers for RT-PCR analysis were provided in Table 1. Relative mRNA abundance of genes of interest was standardized to the abundance of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA and calculated by ΔΔCt value. For comparison between FL-SPAK, KS-SPAK, OSR1, and Dnpep, the efficiency of each primer set in RT-PCR assay was calculated from the results of three serial (4-, 16-, 64-fold) dilutions of cDNA, which encompass our working dilution (~10-fold dilution) (17). The slope of threshold cycles obtained from FL-SPAK, KS-SPAK, OSR1, and Dnpep, and GAPDH reactions of serially diluted sample tubular cDNA was used to calculate the corresponding efficiencies (E) according to the equation: E = 10^{-1/ΔCt}. To analyze the ratio of FL-SPAK to KS-SPAK and the fold change of SPAK isoforms, the mRNA level of SPAK isoforms in each segment was standardized to the level of FL-SPAK in cTAL (defined as 1). Each sample was assayed in triplicate.

In vitro microperfusion, sodium flux, and transepithelial potential difference. After the mouse was killed, the kidney was removed quickly, sliced in thin coronal sections, and placed in Hanks’ solution containing (in mM) 137 NaCl, 5 KCl, 0.8 MgSO4, 0.33 Na2HPO4, 0.44 KH2PO4, 1 MgCl2, 10 tris (hydroxymethyl) amino methane hydrochloride, 0.25 CaCl2, 2 glutamine, and 2 l-lactate at 4°C. The medullary or cortical thick ascending limb of Henle’s loop (mTAL or cTAL) was then dissected free hand without collagenase and transferred to a 1-ml temperature-controlled bathing chamber. Tubules were perfused in vials as previously described (5).

Isolated TALs were perfused at a rate of ~5 nl/min. The perfusate contained (in mM) 115 NaCl, 25 NaHCO3, 2.3 Na2HPO4, 10 Na acetate, 1.8 CaCl2, 1 MgSO4, 5 KCl, 8.3 glucose, and 5 alanine and had an osmolality equal to that of the bathing solution that contained 6 g/dl of albumin. There were at least three measurements of the perfusion and the collected tubular fluid in each experiment. At the end of the experiment, furosemide purchased from Sigma was used to block NKCC2-mediated Na⁺ reabsorption. Na⁺ transport (Jnα) was calculated using the equation: Jnα (pmol·min⁻¹·mm⁻¹) = ([Na]perfusate − [Na]collectate)Vf/JL, where Vf is collection rate (~5 nl/min) and L is the tubular length (0.4–0.8 mm). The transepithelial potential difference (PDTE) was determined using the perfusion pipette as a bridge into the tubular lumen and referenced to the bathing solution using a Keithley 6517A programmable electrometer (Cleveland, OH). To test the effect of vasopressin, bath solution containing 10⁻¹² M vasopressin analog desmopressin (DDAVP) was added to the bathing chamber and exchanged at a rate of 0.5 ml/min. After the steady-state positive PDTE was achieved, three timed collections of tubular fluid were collected in a calibrated constant volume pipette. Na⁺ concentrations of perfusate and collected fluid were measured using a Na⁺-selective electrode (Sodium Ionophore II-Cocktail A, Fluka) as previously described (4).

Statistical analysis. All results are expressed as means ± SE. Difference between groups was assessed using Student’s t-test. A P value <0.05 (*) was considered to be statistically significant.

RESULTS

Characterization of SPAK isoforms, OSR1, and Dnpep mRNA expression in renal tubules. McCormick et al. (12) reported that KS-SPAK, which is a kidney-specific alternatively spliced isoform of SPAK with a unique exon 5a and lacks NH2-terminal amino acids including the majority of kinase domain, functions as an antagonist of the kinase activity of FL-SPAK. They also reported that KS-WNK1 is relatively more abundant in the medulla while FL-SPAK is more abundant in the cortex. Markadieu et al. (11) recently demonstrated that the smaller fragments of SPAK could be the proteolytic

Table 1. Primer sequences for RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence (5′-3′)</th>
<th>Orientation</th>
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<tr>
<td>FL-SPAK</td>
<td>GCTGTCGACGAGAAGGCGGAGA</td>
<td>Sense</td>
</tr>
<tr>
<td>KS-SPAK</td>
<td>GTGTCGACGAGAAGGCGGAGA</td>
<td>Antisense</td>
</tr>
<tr>
<td>OSR1</td>
<td>TAAATGCAGACGAGAAGGCGGAGA</td>
<td>Sense</td>
</tr>
<tr>
<td>DNPEP</td>
<td>CAGGAATTCGCTCGATGGGAGGA</td>
<td>Antisense</td>
</tr>
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<td>Antisense</td>
</tr>
<tr>
<td>AQP2</td>
<td>TGGTGTCTGATGGAAGATG</td>
<td>Sense</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGCCTCCGATGAAATAATGGT</td>
<td>Antisense</td>
</tr>
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FL-SPAK, full-length STE20/SPS1-related proline/alanine-rich kinase; KS-SPAK, kidney-specific SPAK; OSR1, oxidative stress-responsive kinase-1; DNPEP, aspartyl aminopeptidase; NKCC2, Na⁺-K⁺-2Cl⁻ cotransporter; NCC, NaCl cotransporter; AQP2, aquaporin-2.
products of zinc metalloprotease Dnpep. We measured relative mRNA abundance of FL-SPAK, KS-SPAK, OSR1, and Dnpep in renal tubules including the proximal tubule (PT), mTAL and cTAL, distal convoluted tubule (DCT), connecting tubule (CNT), and cortical collecting duct (CCD) using quantitative RT-PCR. Identification of individual tubular segments was based on the morphology and relative location and confirmed by measuring the mRNA abundance of nephron segment-specific markers (NKCC2 for TAL, NCC for DCT, aquaporin-2 for CNT and CCD) (Fig. 1A) (5). To allow direct comparison of abundance of these SPAK-related genes, we first determined the efficiencies of PCR primers (see Table 1) used in the RT-PCR assays (position of primers is shown in Fig. 1B). The calculated efficiencies of FL-SPAK, KS-SPAK, OSR1, Dnpep, and GAPDH RT-PCR assays were very close (FL-SPAK: 1.94, KS-SPAK: 1.91, OSR1: 1.93, Gnpep: 1.92, GAPDH: 1.97, Fig. 1C). The similar efficiencies between RT-PCR assays support the validity of direct comparison of mRNA abundance despite using different primer sets (17).

The expression of FL-SPAK was most abundant in the mTAL and relatively less abundant in PT and tubules downstream of mTAL (Fig. 2A, left). Quantitatively, the level in mTAL was about one- and twofold higher than those in cTAL and DCT, respectively. Compared with FL-SPAK, the mRNA expression of KS-SPAK was relatively lower (Fig. 2B, left). The ratio of FL-SPAK to KS-SPAK mRNA was consistently greater than 1 in all tubular segments, with a relatively lower ratio in CNT (2.1) and CCD (1.6) compared with the mTAL (12.3), cTAL (12.5), and DCT (10.2) (Fig. 2A, inset). Thus, FL-SPAK is the major SPAK mRNA throughout renal tubules, especially in TAL and DCT. The mRNA for SPAK isoforms was barely detectable in tubules isolated from SPAK-KO mice (Fig. 2A, B, right), validating the specificity of qRT-PCR assay for detecting SPAK gene transcripts.

The mRNA for OSR1 was present in all renal tubular segments, and the pattern of expression among renal tubules was more evenly distributed than that for SPAK isoforms (Fig. 2C, left). The overall level of expression of OSR1 in tubules was about two- to sevenfold lower than that of FL-SPAK. Levels of mRNA for OSR1 in SPAK-KO tubules were not significantly different from that in WT tubules (Fig. 2C). The mRNA for Dnpep was also present in all tubular segments including PT in which SPAK expression had very low abundance (Fig. 2D, left). The mRNA expression of Dnpep was not altered in SPAK-KO tubules relative to that in WT tubules (Fig. 2D).

**Fig. 1. Purity of dissected tubules confirmed by measuring mRNA level of tubule-specific marker and direct comparability of mRNA level of STE20/SPS1-related proline/alanine-rich kinase (SPAK)-related genes. A: selective expression of Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC2) in medullary thick ascending limb (mTAL) and cortical TAL (cTAL), of NaCl cotransporter (NCC) in distal convoluted tubule (DCT), and of aquaporin-2 (AQP2) in connecting tubule (CNT) and cortical collecting duct (CCD); n = 12 tubules for each. B: schematic representation of targeted gene exons showing the positions of primers (arrows) used for RT-PCR analysis of full-length SPAK (FL-SPAK), kidney-specific SPAK (KS-SPAK), oxidative stress-responsive kinase-1 (OSR1), and aspartyl aminopeptidase Gnpep in this study. C: similar efficiencies of SPAK-related gene RT-PCR assays. Wild-type cTAL cDNA was serially diluted one-quarter (4-, 16-, 64-fold dilution), and 3 μL of each dilution were used in the PCR assays. The threshold cycle was measured and plotted against the log of the dilution. The slope (bold letter) of linear regression was used to calculate the efficiency of RT-PCR.**
tion in cTAL and mTAL isolated from SPAK-KO mice and their control littersates. In WT cTALs, the Na⁺ reabsorption rate (J_{Na⁺}) was at 165 ± 4.2 pmol·min⁻¹·mm⁻¹ (Fig. 3A, left bar). This reabsorption rate agrees with previous reports in cTAL (5). In cTALs isolated from SPAK-KO mice, the mean Na⁺ reabsorptive flux was 57 ± 3.8 pmol·min⁻¹·mm⁻¹, about one-third of level of control mice (Fig. 3A, right bar, P < 0.0001). The lumen-positive PD_{TE} in WT and SPAK-KO cTALs (9.6 ± 1.2 vs. 4.2 ± 1.1 mV, P = 0.028) exhibited a similar trend with ~60% reduction in SPAK-KO cTAL (Fig. 3B). Addition of furosemide (100 μM) to the luminal perfusate caused ~90% reduction of Na⁺ reabsorption in cTAL (Fig. 3C), indicating that most Na⁺ flux in cTAL is mediated by NKCC2. It is known that there are differences in mTAL compared with the cTAL including the rate and magnitude of NaCl reabsorption and responses to hormones (2). We next measured Na⁺ flux in mTALs isolated from SPAK-KO mice and their control littersates. The Na⁺ reabsorption rate in WT mTAL was 176 ± 15 pmol·min⁻¹·mm⁻¹ (Fig. 4A, left bar). Genetic ablation of SPAK reduced the Na⁺ reabsorption rate to 22 ± 2.4 pmol·min⁻¹·mm⁻¹ (~12.5% of WT; Fig. 4A, right bar, P < 0.0001). Similar to the Na⁺ flux results, lumen-positive PD_{TE} was reduced in SPAK-KO mTAL relative to the WT tubules (4.7 ± 0.6 vs. 10.5 ± 0.5 mV; Fig. 4B, P = 0.004). Similarly, ~90% of Na⁺ reabsorption in mTAL was inhibited by luminal furosemide (Fig. 4C). These results are consistent with the notion that the net effect of SPAK isoforms on NKCC2 in cTAL and mTAL is stimulatory.

**SPAK is not essential for vasopressin stimulation of NKCC2.** Vasopressin enhances NKCC2 activity (10). Whether SPAK is involved in the signaling cascade of vasopressin-induced NKCC2 activation is unknown. We compared the response of Na⁺ reabsorption with vasopressin in mTALs and cTALs isolated from SPAK-KO and WT mice. Addition of 10⁻¹⁰ M vasopressin analog desmopressin (DDAVP) to bath solution enhanced the lumen-positive PD_{TE} and reached a steady-state PD_{TE} after 20–30 min (not shown in graph in Fig. 5). DDAVP increased J_{Na⁺} in WT mTAL as well as cTAL by ~80% (Fig. 5: 176 ± 15 to 298 ± 8 pmol·min⁻¹·mm⁻¹, P < 0.0001 and 165 ± 4.2 to 297 ± 9.5 pmol·min⁻¹·mm⁻¹, P < 0.0001, respectively). DDAVP increased J_{Na⁺} in SPAK-KO mTAL and cTAL by 2.5- to 4-fold (22 ± 2.4 to 89 ± 7 pmol·min⁻¹·mm⁻¹, P < 0.0001
and 57 ± 3.8 to 138 ± 16.7 pmol·min⁻¹·mm⁻¹, P < 0.0001, respectively).

**DISCUSSION**

Studies using indirect assessment of NKCC2 activity (such as protein abundance and phosphorylation and sensitivity to furosemide) in several SPAK loss-of-function mutant mouse models have produced apparent discrepant results pertaining to the effect of SPAK on Na⁺ transport in the TAL (12, 21, 30). Rafiqi et al. (21) found that levels of total and phosphorylated NKCC2 were both decreased in knock-in mice carrying homozygous loss-of-function SPAK²⁴₃A alleles. These results support the notion that SPAK stimulates NKCC2 in TAL, which is consistent with results of studies by others showing that SPAK interacts and activates NKCC2 transporter (18, 19). In contrast, Yang et al. (30) showed that the abundance of phosphorylated NKCC2 was increased in SPAK-KO mice created by deletion of exon 9 and 10 of SPAK gene. Yang et al. proposed that OSR1 is upregulated in TAL of SPAK-KO mice to activate NKCC2 in the absence of SPAK. Similar to the results of study by Yang et al., McCormick et al. (12) reported increased abundance of both total and phosphorylated NKCC2 in a separate mouse model of SPAK-KO mice created by gene-trap insertion. McCormick et al. also reported identification of the smaller KS-SPAK isoform that inhibits the function of FL-SPAK in vitro. Because both SPAK-KO mouse models generated by deletion of exons 9 and 10 and by gene-trap insertion are expected to inactivate both FL-SPAK and KS-SPAK, McCormick et al. suggested that the relative abundance of KS-SPAK over FL-SPAK in TAL explains the results of net inhibition of NKCC2 by SPAK isoforms observed by Yang et al. and in their own studies. This notion yet cannot fully explain the finding by Rafiqi et al. because knock-in of SPAK²⁴₃A allele should inactivate both FL-SPAK and KS-SPAK as well.

The purpose of our study was to examine the role of SPAK in Na⁺ transport in TAL by direct measurement of the Na⁺ reabsorption rate using in vitro microperfusion of isolated TAL from SPAK-KO and control mice. In the study, we also measured the mRNA abundance in isolated tubules of FL-SPAK, KS-SPAK, OSR1, and the metalloprotease Dnpep that has been suggested to play a role in generation of potentially inhibitory smaller SPAK fragments. Our results demonstrate that the transcript for FL-SPAK is more abundant than that for KS-SPAK throughout the nephron segment from PT to CCD. The differential expressions of FL-SPAK over KS-SPAK in mTAL, cTAL, and DCT (where the in vivo targets of SPAK: NKCC2 and NCC are expressed) are striking (more than 10-fold). While the abundance of mRNA does not necessarily reflect the abundance of protein, the overwhelming difference in the mRNA suggests that FL-SPAK protein is likely more abundant than KS-SPAK protein in TAL. We also found that mRNA for OSR1 is significantly less than mRNA for FL-SPAK in TAL. Moreover, mRNA for OSR1 is not upregulated...
in SPAK-KO mice in our experimental setting, indicating that OSR1 is unable to fully compensate for the loss of SPAK. The mRNA for Dnpep is present in all renal tubular segments examined. Its expression is not significantly altered in SPAK-KO mice vs. WT mice. In the microperfusion study, we show that Na⁺ reabsorption in mTAL and cTAL is markedly reduced in SPAK-KO mice compared with WT mice. The SPAK-KO mice we used in the study are homozygous for deletion of exons 9 and 10 of the SPAK gene and evidently lack both FL-SPAK and KS-SPAK transcripts in renal tubules (30). Our results, thus, provide compelling evidence that the combined net effect of SPAK isoforms on Na⁺ reabsorption in TAL is stimulatory.

Others have also reported the existence of multiple SPAK isoforms. Piechotta et al. (18) first reported multiple bands of SPAK in Western blotting using SPAK-specific antibody recognizing the COOH-terminal epitope. They found that a second kozak consensus sequence may provide an alternative translation initiation and responsible for one of the smaller truncated SPAK proteins. Recently, Grimm et al. (7) reported two smaller forms of SPAK found prominently in the kidney. The larger one was named SPAK2 and had the molecular size closer to that predicted for the second initiation site. The smaller one was believed to correspond to the KS-SPAK reported by McCormick et al., which was generated by alternative transcription initiation from within a kidney-specific unique exon 5a. The relative abundance and distribution vary between studies by McCormick et al. and Grimm et al. The former reported that FL- and KS-SPAK were preferentially expressed in renal cortex and medulla, respectively (12). Grimm et al. (7) reported that FL-SPAK, while the major SPAK isoform in mouse renal cortex, was present in the medulla with nearly equal amount compared with the smaller SPAKs. Moreover, SPAK2, rather than KS-SPAK, was the larger SPAK isoform in the kidney. In addition, Nguyen et al. (15, 16) showed that FL-SPAK was the major SPAK isoform in rat renal medulla and KS-SPAK and SPAK2 were barely detectable therein.

Overall, it remains difficult to compare results of all existing studies. Due to limitation of specific antibodies for individual isoforms and inadequate understanding of promoter structure and differential transcription, it is not possible to know for certain at the moment whether multiple bands of SPAK detected by Western blot arise from alternative translation, post-translational modification, and/or degradation. Our knockout mice are generated by deletion of exon 9 and 10, thus most, if not all, of the NH₂-terminal SPAK isoforms (FL-SPAK, SPAK2, KS-SPAK) are inactivated in our study. It is important to note that, while our results support that in normal mouse in baseline the combined net effect of SPAK isoforms stimulates Na⁺ reabsorption in TAL, they cannot differentiate the individual effect of specific SPAK isoforms. The role of individual SPAK isoforms or their ratios in TAL (and DCT as well) is likely more complex and requires further investigation. Animal models with conditional knockout of KS-SPAK or Dnpep will be helpful to address the in vivo function of these smaller SPAKs.

The transcellular Na⁺ reabsorption in TAL is a multi-step process that involves cellular uptake of Na⁺ and chloride across the apical membrane predominantly mediated by NKCC2 and exits through the basolateral membrane via transporters such as Na⁺-K⁺-ATPase and Cl⁻ channel. Potassium transported through NKCC2 is recycled across the apical membrane via ROMK resulting in a lumen-positive PD_TEL. The lumen-positive PD_TEL provides driving force for the paracellular reabsorption of cations. Na⁺-H⁺ exchanger is also present in the apical membrane of the TAL but contributes to only a small part of cellular Na⁺ uptake for the transcellular Na⁺ reabsorption. Overall, the transepithelial Na⁺ reabsorption in TAL is predominantly (~90%) dependent on NKCC2, a process that generates lumen-positive PD_TEL. Our finding that SPAK knockout decreases Na⁺ reabsorption and PD_TEL to a similar extent indicates that the main SPAK target for Na⁺ reabsorption in TAL is NKCC2-dependent.

Our present finding that NKCC2-mediated Na⁺ reabsorption is decreased in SPAK-KO mice does not apparently agree with the conclusion by Yang et al. and by McCormick et al. (12, 30) that NKCC2 activity is increased in SPAK-KO mice, which is predominantly based on results that phosphorylated NKCC2 is increased in SPAK-KO mice. It should be noted that the NKCC2 gene is alternatively spliced and there are as many as six different NKCC2 isoforms in mice (9, 20). These splice variants differ in the COOH-terminus have the same NH₂-terminal amino acids that will be recognized by anti-phospho-NKCC2 antibodies. It is believed that some splice variants are inactive and some may exert dominant-negative inhibition on the full-length variant (9, 20). Thus, the level of phosphorylated NKCC2 might not be used as a sole indicator of NKCC2 activity. Moreover, it is also conceivable that SPAK may also regulate other transporters in TAL critical for NKCC2-mediated Na⁺ reabsorption such as ROMK channel, Cl⁻ channel, and Na⁺-K⁺-ATPase. While the effects of SPAK on NKCC2 and these other transporters cannot be distinguished, our present study directly measures NKCC2-mediated Na⁺ reabsorption and provides the most physiologically relevant function of SPAK in TAL in vivo. Finally, it should be noted that our study does not measure phosphorylation of NKCC2 and is not intended to address the role of phosphorylation in the function of NKCC2.

A recent study reported that angiotensin II infusion caused hypertension and natriuresis in rats by differentially regulating NKCC2 in mTAL and cTAL (15). The pattern of SPAK regulation mimicked the pattern of NKCC2 phosphorylation, indicating that the activity of NKCC2 in TAL is positively correlated with the level and activity of SPAK. In our study, the effect of SPAK knockout on Na⁺ reabsorption is more pronounced in mTAL than cTAL, consistent with the finding that higher FL-SPAK level in mTAL. Our study does not address the mechanism for SPAK regulation of NKCC2. Reiche et al. (22) previously reported that SORLA (sorting protein-related receptor with A-type repeats) regulates the luminal trafficking of SPAK in TAL. This is known to vasopressin stimulates phosphorylation and luminal trafficking of NKCC2 via the PKA signaling pathway (10). The potential role for other protein kinases has also been raised (8). We found that vasopressin analog DDAVP at a physiologically relevant concentration enhances Na⁺ reabsorption in both WT and SPAK-KO TAL. Because the baseline Na⁺ reabsorption rate in SPAK-KO TAL was much lower than that in the WT tubule, it is difficult to know whether the responses to vasopressin were appropriate. Nonetheless, our data indicate that SPAK is not essential for vasopressin-induced NKCC2 activation. A recent study also showed that...
vasopressin increased the NKCC2 phosphorylation and surface expression in both WT and SPAK gene-trap mice (24). Taken together, the vasopressin-induced NKCC2 activation is likely not solely mediated by SPAK.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: C.-J.C., J.Y., M.B., and C.-L.H. conception and design of research; C.-J.C. and J.Y. performed experiments; C.-J.C., J.Y., M.B., and C.-L.H. analyzed data; C.-J.C., J.Y., M.B., and C.-L.H. interpreted results of experiments; C.-J.C., J.Y., C.-L.H. prepared figures; C.-J.C. and C.-L.H. drafted manuscript; C.-J.C., M.B., and C.-L.H. edited and revised manuscript; C.-J.C., J.Y., M.B., and C.-L.H. approved final version of manuscript.

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