WNK kinases are serine-threonine kinases with an atypical placement of the catalytic lysine. WNK1, the first member discovered, has multiple alternatively spliced isoforms, including a ubiquitously expressed full-length long form (L-WNK1) and a kidney-specific form (KS-WNK1) predominantly expressed in the kidney. Intronic deletions of WNK that increase WNK1 transcript cause pseudohypoaldosteronism type 2, an autosomal-dominant disease characterized by hypertension and hyperkalemia. L-WNK1 inhibits renal K\(^+\) channel ROMK, likely contributing to hyperkalemia in PHAII. Previously, we reported that KS-WNK1 by itself has no effect on ROMK1 but antagonizes L-WNK1-mediated inhibition of ROMK1. Amino acids 1–253 of KS-WNK1 (KS-WNK1(1–253)) are sufficient for reversing the inhibition of ROMK1 caused by L-WNK1(1–491). Here, we further investigated the mechanisms by which KS-WNK1 counteracts L-WNK1 regulation of ROMK1. We reported that two regions of KS-WNK1(1–253) are involved in the antagonism of L-WNK1; one includes the first 30 amino acids unique for KS-WNK1 encoded by the alternatively spliced initiating exon 4A, and the other is equivalent to the autoinhibitory domain (AID) of L-WNK1. Mutations of two phenylalanine residues known to be critical for autoinhibitory function of AID abolish the ability of the AID region of KS-WNK1 to antagonize L-WNK1. To examine the physiological role of KS-WNK1 in the regulation of renal K\(^+\) secretion, we generated transgenic mice that overexpress amino acids 1–253 of KS-WNK1 under control of a kidney-specific promoter. Transgenic mice have higher serum K\(^+\) levels and higher urinary fractional excretion of K\(^+\) compared with wild type littermates despite the same amount of daily urinary K\(^+\) excretion. Moreover, transgenic mice (compared with wild type littermates) displayed a higher abundance of ROMK on the apical membrane of distal nephron. Thus, KS-WNK1 is an important physiological regulator of renal K\(^+\) excretion, likely through its effects on the ROMK1 channel.

WMK (with no lysine (K)) kinases are a recently discovered family of large serine-threonine protein kinases characterized by an atypical placement of the catalytic lysine (1). There are four family members, WNK1–4 (1–3), each encoded by a separate gene. WNK1 protein is over 2,100 amino acids long and contains an ~270-amino acid kinase domain located near the amino terminus (1). WNK2, WNK3, and WNK4 are between 1,200 and 1,600 amino acids in length (1–3). The four WNK kinases share a conserved kinase domain with 85–90% sequence identity, an autoinhibitory domain (AID),\(^2\) one or two coiled-coiled domains, and multiple PXXP motifs for potential protein-protein interaction (1–3). Beyond these conserved domains and motifs, amino acid sequences of WNK1–4 are divergent. The human WNK1 gene spans more than 150 kb in chromosome 12 and consists of 28 exons (3). A WNK1 transcript produced from all 28 exons (encodes a peptide referred to herein as long WNK1 (L-WNK1)) is ubiquitously expressed (1). A shorter WNK1 transcript produced by an alternative 5’ exon (exon 4A) and exon 5–28 is expressed exclusively in the kidney, encoding a peptide known as kidney-specific WNK1 (KS-WNK1) (4, 5). KS-WNK1 is ~1,700 amino acids in length and lacks amino acids 1–437 of the long WNK1 that are encoded by exon 1–4. The first 30 amino acids of KS-WNK1 are encoded by exon 4A (4, 5) and unique to KS-WNK1. In the kidney, KS-WNK1 is predominantly in the distal convoluted tubule, the connecting tubule, and the cortical collecting duct (6), suggesting a role in these segments. Analysis by real time PCR reveals that the transcript for KS-WNK1 in kidney is more abundant than that for L-WNK1 (4, 5). The relative protein abundance of KS-WNK1 versus L-WNK1 has not been determined. Alternate splicing of exons 11 and 12 also occurs and produces isoforms with peptide length between that of L-WNK1 and KS-WNK1 and with differential tissue distributions (4, 5).

Large deletions within the first intron of WNK1 increase the abundance of WNK1 transcript and cause pseudohypoaldosteronism type 2 (PHAII; also known as familial hyperkalemic hypertension or Gordon syndrome), an autosomal-dominant disorder featured by hypertension and hyperkalemia (3, 7). Many studies have examined the role of WNK kinases in the regulation of renal ion transport (8–11). With respect to WNK1 on K\(^+\) transport, it was reported that L-WNK1 decreases cell surface abundance of renal K\(^+\) channel ROMK1 (renal outer medullary potassium (K) channel 1) by increasing clathrin-coated vesicle-mediated endocytosis of the channel

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\(^2\) The abbreviations used are: AID, autoinhibitory domain; L-WNK1, long form of WNK1; KS-WNK1, kidney-specific form of WNK1; HCK, human embryonic kidney; PHAII, pseudohypoaldosteronism type II; TG, transgenic; PBS, phosphate-buffered saline; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase.
(12, 13). ROMK K⁺ channels are expressed in the connecting tubule and the cortical collecting duct and are important for base-line (non-flow-stimulated) renal K⁺ secretion (14). Thus, a decrease in K⁺ secretion by the kidney resulting from the inhibition of ROMK by L-WNK1 may contribute to hyperkalemia in patients of PHAII with WNK1 mutations. However, there are multiple alternatively spliced WNK1 isoforms differentially expressed in tissues (4, 5). The effects of deletions of the first intron on splice variants of WNK1 and the effects of individual isoforms on K⁺ transport remain largely unknown.

Recently, we and others reported that kidney-specific WNK1, by itself, has no effect on ROMK1 but antagonizes the inhibition of ROMK1 caused by L-WNK1 (12, 15). K⁺ secretion by kidney is critical for controlling serum K⁺ levels and overall K⁺ homeostasis (14). As an important pathway for K⁺ secretion in kidney, the abundance of ROMK on the apical membrane of distal nephron is regulated by dietary K⁺ intake (14). The apical ROMK abundance decreases or increases during low or high dietary K⁺ intake, respectively (16, 17). The decrease in the apical abundance of ROMK in response to dietary K⁺ restriction involves an increase in the clathrin-mediated endocytosis and subsequent degradation of the channel protein (18, 19). We reported that dietary K⁺ restriction in rats increases the expression of L-WNK1 and decreases that of KS-WNK1 (12). The increase in the L-WNK1 to KS-WNK1 ratio would be expected to cause inhibition of ROMK1. These results suggest that KS-WNK1 is an important physiological antagonist of L-WNK1, and the ratio of L-WNK1 to KS-WNK1 regulates surface abundance of ROMK1 and renal K⁺ secretion during changes in dietary K⁺ intake.

In the present study, we further examined the mechanism by which KS-WNK1 antagonizes L-WNK1 regulation of ROMK1. We identified two regions within amino acids 1–253 of KS-WNK1 that are involved in binding to and antagonism of WNK1 and found that they display lower serum K⁺ levels and increased tubular excretion of K⁺ relative to wild type littermates despite a similar K⁺ intake. These results further support the important physiological role of KS-WNK1 in the regulation ROMK1 activity and renal K⁺ excretion.

**EXPERIMENTAL PROCEDURES**

**Plasmid DNA Constructs**—PEGFP-ROMK1, pCMV-Myc-WNK1(1–491), and pIRES-FLAG-KS-WNK1(1–253) were described previously (12). WNK1 fragments were amplified by PCR using rat WNK1 cDNA as the template and subcloned into a pCMV5-Myc vector. Fragments of rat KS-WNK1 were amplified by PCR and subcloned into a carboxyl-terminal FLAG vector (pIRES-hrGFP-1a) (Stratagene). Point mutations were generated by site-directed mutagenesis (QuickChange kit; Stratagene) and confirmed by sequencing.

**Cell Culture, Immunoprecipitation, and Western Blot Analysis**—HEK 293 cells were cultured, transfected, and harvested as described previously (12). For immunoprecipitation, the proteins were immunoprecipitated from cell lysates by using monoclonal anti-FLAG antibody (1:100 dilution; Sigma) and followed by protein A-Sepharose beads. The precipitates were washed three time with 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100. For Western blot analysis, total lysates, immunoprecipitates, or kidney homogenates were resolved by SDS-PAGE gel electrophoresis, and proteins were transferred onto nitrocellulose membranes. The membranes were incubated with the indicated antibodies and developed using enhanced chemiluminescence.

**Whole Cell Patch-Clamp Recording of ROMK1 Channels**—HEK 293 cells were cotransfected with CDNAs encoding GFP-ROMK1 and a fragment of L-WNK1 and/or KS-WNK1. In each experiment, the total amount of DNA for transfection was balanced by using empty vectors. Approximately 36–48 h after transfection, whole cell currents were recorded by using an Axopatch 200B amplifier as previous described (12). Transfected cells were identified by using epifluorescent microscopy. The bath and pipette solution contained 145 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes (pH 7.4), and 145 mM KCl, 2 mM EDTA, 10 mM Hepes (pH 7.4), respectively. Capacitance and access resistance were monitored and 75% compensated. The voltage protocol consists of 0-mV holding potential and 400-ms steps from −100 to 100 mV in 20-mV increments.

**Generation of Transgenic Mice**—The FLAG-KS-WNK1(1–253) fusion fragment was generated by PCR using plasmid pIRES-hrGFP-KS-WNK1(1–253) as template. The ~0.8-kb restriction fragment was isolated and cloned into unique SbfI and Smal sites downstream to the Ksp-cadherin promoter of pKsp-BGH plasmid (provided by Dr. Peter Igarashi, University of Texas Southwestern Medical Center at Dallas). The plasmid insert was verified by DNA sequencing. The ~2.6-kb transgene fragment Ksp–FLAG–KS-WNK1(1–253) was isolated by digestion with Ndel and KpnI followed by agarose gel electrophoresis, electroelution, and purification by anion exchange chromatography (Elutip-d, NH). Purified DNA was concentrated in Microcon 30 filters (Millipore, MA), resuspended at a concentration of 80 ng/μl in microinjection buffer (10 mM Tris-Cl, pH 7.4, 0.25 mM EDTA), and sterilized by filtration through 0.2-μm filters. Transgene DNA was microinjected into the pronuclei of fertilized oocytes by standard pronuclear injection. Fertilized oocytes were from C57BL/6 crosses. Microinjection was performed by the University of Texas Southwestern Transgenic Mouse Core Facility. The microinjected embryos were transferred into the oviducts of pseudopregnant foster mothers and were permitted to develop to term.

**Genotyping of Transgenic Mice**—Founder (G0) mice were identified by PCR analysis. Genomic DNA was isolated from tails of transgenic mice using a standard method. Two pairs of primers were used for genotyping by PCR. One is specific for endogenous mouse WNK1 (forward, 5’-AAA ATA CTC TGT CAG GCT TAA GTG T-3’, and reverse, 5’-TGA AGC CAG GCA TTA AGC ACT C-3’), which would produce a 266-bp fragment in both wild type and transgenic mice. The other is specific for transgenic fragment (forward, 5’-GCA GAT CAG CAT CAA CAG CTG T-3’, and reverse, 5’-CAG ATT TCC TC-3’), which would produce a 320-bp fragment only in transgenic mice. The condition for PCR includes 35 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 45 s. PCR products were detected by electrophoresis on 2.0% agarose gels.
Regulation of ROMK1 by KS-WNK1

A domain structure of L-WNK1 and KS-WNK1 (not drawn in scale). KS-WNK1 lacks the first 437 amino acids of L-WNK1 (encoded by exons 1–4) but contains unique 30 amino acids encoded by an alternatively spliced exon 4A (the first exon of KS-WNK1). The vertical dotted line defines the position of amino acid in L-WNK1 equivalent to amino acid 31 of KS-WNK1. Amino acids of L-WNK1 (438–2126) and KS-WNK1 (31–1719) distal to the dotted line are identical (encoded by exons 5–28). Amino acid 660 of L-WNK1 is equal to amino acid 253 of KS-WNK1. AID, KD, and 4A indicate the autoinhibitory domain, the kinase domain, and the region of KS-WNK1 encoded by exon 4A, respectively. Fragments of KS-WNK1 used in the present study are shown. B, effects of various KS-WNK1 constructs on WNK1(1–491) inhibition of ROMK1. HEK cells were transfected with ROMK1 alone or cotransfected with ROMK1 plus WNK1(1–491) and with KS-WNK1(1–253), KS-WNK1(1–196), KS-WNK1(1–137), or KS-WNK1(1–77), respectively (Fig. 1A). The experiments were repeated three times with similar results.

 immunofluorescent Staining—The mice were anesthetized by Avertin and perfused via the heart with 15 ml of PBS followed by 15 ml of 4% paraformaldehyde in PBS. The kidneys were harvested and postfixed for 4 h in 4% paraformaldehyde in PBS at 4°C, dehydrated by immersion in 30% sucrose in PBS overnight at 4°C, and mounted in OTC (Tissue-Tek) for sectioning. The sections (4–5-μm thickness) were stained with primary antibodies: rabbit polyclonal anti-FLAG antibody (1:300, Sigma) or anti-ROMK1 (1:1000), followed by secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG (1:500) or Alexa Fluor 568 goat anti-rabbit IgG (1:500). The fluorescent images were obtained using Zeiss LSM510 confocal microscope as described (19).

Blood and Urine Measurements—Under anesthesia by Avertin, blood was drawn from mice by retro-orbital bleeding into heparinized tubes. Electrolytes were measured using a flame photometer. Creatinine was measured by capillary electrophoresis (P/ACE MDQ; Beckman Coulter). Spot urine samples were collected by catching spontaneous voids. 24-h urine samples were collected using metabolic cages (Hatteras Instruments). All of the experiments involving animals were performed in compliance with relevant laws and institutional guidelines and were approved by the University of Texas Southwestern Medical Center at Dallas Institutional Animal Care and Use Committee.

Statistical Analysis—Statistical comparisons between two groups of data were made using two-tailed unpaired Student’s t tests. Multiple comparison were made using one-way analysis of variance followed by two-tailed Student’s t tests adjusted for multiple comparisons, p values less than 0.05 and 0.01 were considered significant for single and multiple comparisons, respectively. Experiments shown in each panel of the figures were repeated at least three times with similar results.

RESULTS

Two Regions of KS-WNK1(1–253) Are Involved in the Antagonism of L-WNK1—We have reported that KS-WNK1(1–253) antagonizes inhibition of ROMK1 by L-WNK1 by binding to amino acids 1–491 of L-WNK1 (12). To further define the molecular determinant(s) of KS-WNK1 involved in antagonism of L-WNK1 regulation of ROMK1, we generated several overlapping and nonoverlapping smaller fragments of KS-WNK1(1–253) and examined their effects on WNK1(1–491)-mediated inhibition of ROMK1. These fragments of KS-WNK1 include amino acids 1–196, 1–137, 1–77, and 31–253, respectively (Fig. 1A). To examine the effects of these fragments on antagonism of L-WNK1 regulation of ROMK1, HEK cells were cotransfected with plasmids expressing green fluorescent protein (GFP)-tagged ROMK1, WNK1(1–491) and one each of
KS-WNK1 fragment and recorded for ROMK1 current density using whole cell patch-clamp recording. We have shown that WNK1(1–491) fully recapitulates the effect of full-length L-WNK1 on ROMK1 (12, 13). As shown in Fig. 1B, we confirmed that WNK1(1–491) inhibits ROMK1 (compare bars 1 and 2) and that KS-WNK1(1–253) reverses WNK1(1–491)-mediated inhibition of ROMK1 (bar 3) as reported previously by us (12). Expression of KS-WNK1(1–253) exerts no effect on ROMK1 in the absence of WNK1(1–491) (not shown in Fig. 1B; see Ref. 12). Here, we found that each of the smaller KS-WNK1 constructs generated could reverse the inhibition of ROMK1 caused by WNK1(1–491) (Fig. 1B, bars 4–7).

WNK1 contains an AID just carboxyl-terminal to the catalytic domain that is conserved in WNKs across species (1, 20). The AID domain binds with and suppresses the activity of the kinase domain (20). We have recently shown that AID domain of WNK1 can antagonize WNK1(1–491)-mediated inhibition of ROMK1 (12, 21). Because KS-WNK1(1–253), KS-WNK1(1–196), KS-WNK1(1–137), and KS-WNK1(31–253) each contain the AID domain, it is not surprising that they each can reverse the effect of WNK1(1–491). The ability of KS-WNK1(1–77) to reverse the inhibition of ROMK1 by WNK1(1–491), however, is unexpected and suggests that additional region is involved in interacting with and reversing the effect of WNK1(1–491). KS-WNK1(1–77) contains the unique 30 amino acids encoded by exon 4A. We hypothesized that this region encoded by exon 4A is responsible for reversal of WNK1(1–491)-mediated inhibition of ROMK1 by KS-WNK1(1–77). In this hypothesis, the two regions within KS-WNK1(1–253) (i.e. AID and region encoded by exon 4A) can interact with WNK1(1–491) independently of one another to reverse the effect of WNK1(1–491).

To test this hypothesis, we examined the interaction between WNK1(1–491) and KS-WNK1(1–253), KS-WNK1(1–77) or KS-WNK1(31–253) by coimmunoprecipitation. HEK cells were cotransfected with Myc-tagged WNK1(1–491) and each of FLAG-tagged KS-WNK1 fragments as indicated. As shown in Fig. 1C (bottom panel), anti-FLAG antibody immunoprecipitated FLAG-KS-WNK1(1–77), KS-WNK1(31–253), and KS-WNK1(1–253) (lanes 2–4 indicated by 10-, 29-, and 32-kDa protein bands, respectively). The higher molecular size band (~160-kDa protein band) in lysates of cells expressing KS-WNK1(1–77) (Fig. 1C, lane 2) suggests that KS-WNK1(1–77) may form oligomers. However, we did not observe large molecular size bands for KS-WNK1(1–253) expressed in HEK cells (not shown) nor in transgenic mice (see “Results” below), suggesting that oligomerization of KS-WNK1 does not occur in vivo and thus is not physiologically important. As shown, Myc-WNK1(1–491) (indicated by the 60-kDa protein band in the middle panel of Fig. 1C) coimmunoprecipitated strongly with KS-WNK1(1–253) (Fig. 1C, lane 4) and to a lesser degree with KS-WNK1(1–77) and with KS-WNK1(31–253) (Fig. 1C, lanes 2 and 3). As a control, Myc-WNK1(1–491) did not coimmunoprecipitate with FLAG-tagged pod (Fig. 1C, lane 5), an unrelated transcription factor protein (22). The abundance of input Myc-WNK1(1–491) was not different among experimental groups (Fig. 1C, top panel). These results support the hypothesis that the region encoded by exon 4A (as in “KS-WNK1(1–77)” and the AID domain (as in “KS-WNK1(31–253)”) can interact with WNK1(1–491), independently. Consistently, the interaction with WNK1(1–491) is stronger for KS-WNK1(1–253), which contains both regions. It should be noted that the relatively weaker interaction between WNK1(1–491) and KS-WNK1(1–77) or KS-WNK1(31–253) was sufficient for reversing the inhibitory effect of WNK1(1–491) (Fig. 1B).

Role of AID Domain of KS-WNK1 in the Interaction with L-WNK1 and Regulation ROMK1 Channel—In our recent study reporting that AID reverses WNK1(1–491) inhibition of ROMK1 (21), we did not examine whether the effect was mediated by binding of AID to WNK1(1–491). Here, we examined the binding interaction between WNK1(1–491) and AID domain (as in “FLAG-WNK1(491–555)” and KS-WNK1(1–77) or KS-WNK1(31–253) was sufficient for reversing the inhibitory effect of WNK1(1–491) (Fig. 1B).

Having shown that the AID domain binds to WNK1(1–491) and that phenylalanine residues are critical for binding to and reversal of WNK1(1–491) inhibition of ROMK1, we employed this knowledge to examine the role of AID domain of KS-WNK1(1–253) to reverse the inhibition of ROMK1 by L-WNK1. We found that mutation of AID domain in KS-WNK1(31–253) (KS-WNK1(31–253)/FFAA) prevented it from reversing WNK1(1–491)-mediated inhibition of ROMK1 (Fig. 2C, compare bars 5 and 6). Double phenylalanine mutations of AID domain of KS-WNK1(31–253) did not affect the expression of protein (Fig. 2D, compare lanes 1 and 2). As for L-WNK1(491–555) (Fig. 2B), double phenylalanine mutations of AID slowed the migration of KS-WNK1(31–253) (Fig. 2D, compare lanes 1 and 2). These results support the idea that AID domain within KS-WNK1(1–253) is responsible for reversing the inhibitory effect of WNK1(1–491) inhibition of ROMK1. In contrast, double phenylalanine mutations of the AID domain of KS-WNK1(1–253) did not significantly affect its ability to reverse WNK1(1–491) inhibition of ROMK1 (Fig. 2C, compare bars 3 and 4). These results support the hypothesis that the region encoded by exon 4A (i.e. amino acids 1–30) within KS-WNK1(1–253) is suffi-
Regulation of ROMK1 by KS-WNK1

**FIGURE 2. Role of WNK1 autoinhibitory domain in regulation of ROMK1.** A, partial sequences of L-WNK1 (amino acids 491–555) and KS-WNK1 (amino acids 84–149) surronding the AID domain. B, Myc-WNK1(1–491) and FLAG-WNK1(491–555) or FLAG-WNK1(491–555)/FFAA were immunoprecipitated by anti-FLAG antibody and probing in Western blot analysis by anti-Myc (top panel) or anti-FLAG antibody (bottom panel) as indicated. The experiments were repeated three times with similar results. C, effects of double phenylalanine mutations on KS-WNK1 inhibition of ROMK1. The cells were cotransfected with FLAG-WNK1(491–555) and either KS-WNK1(1–253) (Fig. 3A, bar 3), KS-WNK1(1–77) (Fig. 3A, bar 4), or KS-WNK1(31–253) (bar 5), or KS-WNK1(31–253)/FFAA (bar 6). In addition, we examined the effect of KS-WNK1(1–253) (bar 3), which is the AID domain of L-WNK1 and is identical to the AID domain of KS-WNK1. As shown, we found that none of the above constructs reversed the inhibition of ROMK1 caused by KS-WNK1(1–119) (Fig. 3A). These results are in contrast to results in Fig. 1, which show that KS-WNK1(31–253), KS-WNK1(1–77), and KS-WNK1(31–253) each reversed the inhibition of ROMK1 by KS-WNK1(1–491). Therefore, amino acids 120–491 of L-WNK1 are required for antagonism by KS-WNK1(1–253).

We further tested this hypothesis using biochemical binding assays. The ability of WNK1(1–119) and WNK1(120–491) to interact with AID and region encoded by exon 4A of KS-WNK1 was examined. The cells were transfected with FLAG-tagged KS-WNK1(1–77) (contains exon 4A region) or KS-WNK1(84–148) (contains AID domain) plus Myc-tagged WNK1(1–119), WNK1(1–119), or WNK1(120–491). As shown, KS-WNK1(31–253)/FFAA did not affect the expression of KS-WNK1(1–253) but slowed its migration (Fig. 2D, compare lanes 3 and 4).

**FIGURE 3. Role of amino acids 1–119 versus amino acids 120–491 of L-WNK1 in antagonism of L-WNK1(1–491) by KS-WNK1(1–253).** We have reported that several proline-rich motifs within amino acids 1–119 of L-WNK1 (WNK1(1–119)) are necessary and sufficient for inhibition of ROMK1 (13, 21). Other regions of L-WNK1, including the kinase domain, the AID domain, etc., contribute to the regulation of ROMK1 by direct or indirect modulation of the effect of WNK1(1–119) (12, 21). These findings raise the question of whether KS-WNK1 can antagonize WNK1(1–119) or the antagonism requires amino acids of L-WNK1 beyond 1–119. To answer this question, we cotransfected ROMK1 and WNK1(1–119) with either KS-WNK1(1–253) (Fig. 3A, bar 4), KS-WNK1(1–77) (bar 5), or KS-WNK1(31–253) (bar 6). In addition, we examined the effect of KS-WNK1(1–253) (bar 3), which is the AID domain of L-WNK1 and is identical to the AID domain of KS-WNK1. As shown, we found that none of the above constructs reversed the inhibition of ROMK1 caused by KS-WNK1(1–119) (Fig. 3A). These results are in contrast to results in Fig. 1, which show that KS-WNK1(1–253), KS-WNK1(1–77), and KS-WNK1(31–253) each reversed the inhibition of ROMK1 by KS-WNK1(1–491). Therefore, amino acids 120–491 of L-WNK1 are required for antagonism by KS-WNK1(1–253).

We further tested this hypothesis using biochemical binding assays. The ability of WNK1(1–119) and WNK1(120–491) to interact with AID and region encoded by exon 4A of KS-WNK1 was examined. The cells were transfected with FLAG-tagged KS-WNK1(1–77) (contains exon 4A region) or KS-WNK1(84–148) (contains AID domain) plus Myc-tagged WNK1(1–119), WNK1(1–119), or WNK1(120–491). As shown, KS-WNK1(31–253)/FFAA did not affect the expression of KS-WNK1(1–253) but slowed its migration (Fig. 2D, compare lanes 3 and 4).

As for KS-WNK1(31–253), double phenylalanine mutations did not affect the expression of KS-WNK1(1–253) but slowed its migration (Fig. 2D, compare lanes 3 and 4).
WNK1(1–119) (Fig. 3B, lanes 4–6, respectively). A robust expression of WNK1(1–119) equal or greater than WNK1(1–491) under the same experimental condition has been demonstrated (not shown here; see Refs. 13 and 21). Together, these results support the hypothesis that amino acids 120–491 of L-WNK1 (which include the kinase domain (amino acids 220–491) and N-linker region (amino acids 120–220); see Ref. 21 for more details) are required for exon 4A and AID of KS-WNK1(1–253) to antagonize L-WNK1 inhibition of ROMK1 channel.

**Transgenic Mice Overexpressing KS-WNK1(1–253)—**To investigate the in vivo physiological role of KS-WNK1 in the regulation of K⁺ secretion, we generated transgenic mice overexpressing amino acids 1–253 of KS-WNK1 under the control of a kidney-specific Ksp-cadherin gene promoter, which directs gene expression in the renal tubules from the thick ascending limb to the collecting duct (23). The Ksp-KS-WNK1 transgenic plasmid construct contains FLAG-tagged rat KS-WNK1(1–253) cDNA placed behind the Ksp-cadherin promoter in a pKsp-BGH vector (23). A ~2.6-kb restriction fragment containing Ksp-cadherin promoter, the KS-WNK1(1–253)-FLAG fusion gene, and the polyadenylation signal was purified (Fig. 4A) and used for pronuclear microinjection to generate transgenic founder lines. Genotyping of founder mice was performed by polymerase chain reaction of tail genomic DNA using two sets of PCR primers, one specific for transgene (and produces a 320 bp fragment) and the other specific for endogenous WNK1 (and produces a 266-bp fragment). Fig. 4B shows an example of positive transgene expression in one of founder lines. Founders were mated with wild-type mice of the same genetic background to produce offspring. The relative expression of KS-WNK1 transgene versus endogenous KS-WNK1 in transgenic offspring were examined by quantitative real time PCR using primers containing nucleotide sequence identical for rat and mouse. Fig. 4C shows that the abundance of KS-WNK1 message RNA in mice homozygous for TG(KS-WNK1) is ~3-fold higher than that for wild type mice. Expression of transgenic KS-WNK1(1–253) protein in transgenic but not wild type mice was verified by Western blot analysis of whole kidney homogenates (Fig. 4D).

We further examined the expression and localization of transgenic FLAG-KS-WNK1(1–253) protein in the kidney of homozygous TG(KS-WNK1) mice by immunofluorescent staining using polyclonal anti-FLAG antibodies. Previously, Shao et al. (23) reported that Ksp promoter directs protein expression in renal tubules from the thick ascending limbs to the collecting duct. More recently, Lin and Igarashi3 reported that expression of protein driven by the Ksp promoter (examined using a reporter) is detected in ~20% of proximal tubules and in >90% of tubules from the thick ascending limb to collecting ducts. Consistent with these reports on tubular expression of proteins directed by Ksp promoter, we found that transgenic KS-WNK1(1–253) is abundantly expressed in distal tubular segments of TG(KS-WNK1) (Fig. 5, A and B) but not of wild type mice (Fig. 5, C and D). Importantly, transgenic KS-WNK1(1–253) is abundantly expressed in connecting tubules and cortical collecting ducts, tubular segments where K⁺ secretion occurs predominantly (Fig. 5A, labeled C). Compared with distal tubular segments, the expression in the proximal convoluted tubule is much lower (Fig. 5A, labeled P). Also, the expression of transgenic KS-WNK1(1–253) protein driven by Ksp promoter is abundant in the outer medulla (Fig. 5B), because of a high density of thick ascending limbs and collecting ducts in this region.

3 F. Lin and P. Igarashi, personal communication.
Regulation of ROMK1 by KS-WNK1

Increased ROMK Expression in TG(KS-WNK1) Mice—We next examined the abundance of ROMK in the transgenic mice and wild-type littermates by immunofluorescent staining. ROMK channels are expressed in tubular segments from the thick ascending limb of Henle’s loop to cortical collecting ducts (14, 17). Using an antibody previously characterized by us (19, 24), we found that the expression of ROMK on the apical membrane of tubules is increased in the transgenic mice compared with the wild type (Fig. 6). ROMK channels on the apical membrane of principal cells of connecting tubules and cortical collecting ducts are important exit pathways for base-line (non-flow-stimulated) K+ secretion (14, 17). Higher magnification images revealed that the expression of ROMK on the apical membrane of connecting tubules and cortical collecting ducts is indeed increased in transgenic mice compared with wild type mice (Fig. 6, B and D, labeled C). We do not have a reliable monoclonal anti-FLAG antibody with a low background in the immunofluorescent staining. We therefore used sequential sections (~4–5-μm thickness) for immunofluorescent staining of ROMK and transgenic KS-WNK1 using polyclonal anti-ROMK and anti-FLAG antibodies, respectively. As shown in cortical sections from homozygous TG(KS-WNK1) mice, ROMK (Fig. 6E) and transgenic KS-WNK1 (Fig. 6F) were coexpressed in many distal nephron segments including connecting tubules/cortical collecting ducts (indicated by arrows) and the distal convoluted tubule (indicated by arrowheads).

Renal K+ Excretion in TG(KS-WNK1) Transgenic Mice—We used the transgenic model to study the role of KS-WNK1 in K+ homeostasis and renal K+ secretion. We found that serum K+ levels were significantly lower in homozygous TG (KS-WNK1) than in wild type littermates (3.9 ± 0.2 mM versus 4.9 ± 0.2 mM, p = 0.02) (Fig. 7A). A decrease in serum K+ levels may be due to decreased dietary intake, increased intracellular shift, and/or increased net secretion by renal tubules. To examine these possibilities, we measured 24-h urinary excretion and fractional excretion of K+ (Fek) in homozygous TG(KS-WNK1) and wild type mice. As shown, the steady-state 24-h urinary K+ excretion was not different between TG and wild type mice (Fig. 7B), suggesting that the two groups have equal dietary K+ intake. Fractional excretion of K+ (Fek), however, were much higher in homozygous TG(KS-WNK1) than in wild type (Fig. 7C, 11.5 ± 0.84% versus 5.1 ± 1.67%, p = 0.01). 24-h urinary volume, creatinine excretion, and creatinine clearance were not different between TG and wild type littermates (not shown). These results support the idea that the decrease in serum K+ levels in TG mice is due to increased net secretion of K+ by renal tubules. For comparison, steady-state serum Na+ (155 ± 3 mM versus 155 ± 4 mM), 24-h urinary Na+ excretion (147 ± 12 μM versus 177 ± 15 μM), and fractional excretion of Na+ (1.7 ± 0.3% versus 2.2 ± 0.4%) were not significantly different between homozygous TG mice and wild type littermates (n = 8 each group; p > 0.1 for all).

DISCUSSION

WNK kinases comprise a recently identified group of serine and threonine kinases in which the location of the lysine required for ATP binding is unique (1). Mutations in the genes encoding two members, WNK1 and WNK4, cause PHAII, a disease characterized by hypertension and hyperkalemia (3, 7). WNK4 is expressed primarily in epithelial tissues including the kidney (25). WNK1 has multiple isoforms including a ubiquitously expressed L-WNK1 and a kidney-specific KS-WNK1 (4, 5). Many studies have reported that L-WNK1 and WNK4 play important roles in regulating renal electrolyte homeostasis (8–11). The function and the physiological role of KS-WNK1 are relatively less understood.

We have recently shown that KS-WNK1 is an antagonist of L-WNK1 regulation of ROMK1 and that amino acids 1–253 of KS-WNK1 are sufficient for the antagonism of L-WNK1 (12).
In the present study, we further report that two regions within amino acids 1–253 of KS-WNK1 are critical for antagonism of L-WNK1. One is the region encoded by the alternative initiating exon 4A. This region is unique to KS-WNK1. The other is the region equivalent to the AID domain of L-WNK1. The AID domain of WNK1 contains a FXF motif that suppresses the catalytic activity by direct binding to the kinase domain (20). Mutation of phenylalanine residues releases the inhibition of WNK1 kinase activity by the AID domain (20). The FXF motif is reminiscent of one type of ERK2 docking domain found in several proteins (26). Interestingly, the AID of WNK1 inhibits kinase activity of ERK2 (20).

The regulation of ROMK1 by WNK1 involves protein–protein interactions that are independent of WNK1 kinase activity (13, 21). The kinase and AID domains of WNK1, nevertheless, play critical roles in its regulation of ROMK1 (13, 21). To understand the role of WNK1 kinase domain in the regulation of ROMK1, we have found that proper folding of the WNK1 kinase domain (not its kinase activity) is important for the amino-terminal proline-rich motifs of WNK1 to bind the endocytic scaffold protein intersectin (13, 21). Binding of WNK1 to intersectin leads to enhanced endocytosis (and thus inhibition) of ROMK. We have further shown that AID domain contributes to WNK1 inhibition of ROMK1 by binding and interfering with the function of the kinase domain (12, 13, 21). Amino acids 84–148 of KS-WNK1 are identical to the AID domain of L-WNK1 (amino acids 491–556) (Fig. 1A). Thus, it is not surprising that this AID-equivalent region of KS-WNK1 can antagonize L-WNK1 inhibition of ROMK1. These findings, however, are interesting in light of the fact that KS-WNK1 does not contain a kinase domain. This fact supports the idea that the physiological role of AID domain of KS-WNK1 is to regulate other WNK kinases that contain the kinase domain, such as L-WNK1. It would be interesting to investigate in the future whether KS-WNK1, through its AID domain, also regulates other WNKs and/or other protein kinases with a FXF motif in the kinase-inhibitory domain as in ERK2.

To examine the in vivo role of KS-WNK1 in the regulation of K⁺ secretion, we generated transgenic mice overexpressing KS-WNK1(1–253) in kidney tubules. KS-WNK1(1–253)-transgenic mice have lower serum K⁺ levels and increased fractional excretion of K⁺ compared with wild type littermates despite the same level of dietary K⁺ intake. Compared with the wild type mice, the expression of ROMK in the apical membrane of renal tubules including connecting tubules and cortical collecting ducts is markedly increased in the transgenic mice. These results support the hypothesis that KS-WNK1 antagonizes the L-WNK1-mediated enhancement of endocytosis of ROMK1. Overall, these results support the idea that KS-WNK1 is a physiological antagonist of L-WNK1 with respect to renal K⁺ excretion. Yet, there are limitations in our study that deserve caution. First, endogenous KS-WNK1 transcript is detected in the thick ascending limb, the distal convoluted tubule, and the cortical collecting duct but not in the proximal convoluted tubule (6). As above, TG-KS-WNK1(1–253) driven by the exogenous Ksp promoter is weakly expressed in the proximal tubule in addition to its abundant expression in the distal nephron segments. The possibility that a low level expression of TG-KS-WNK1(1–253) in the proximal tubule may affect K⁺ transport via nonspecific mechanism cannot be excluded. Second, we used amino acids 1–253 of KS-WNK1 for transgenic expression in mice because this region is sufficient for antagonism of L-WNK1. Our results, although supporting the idea that amino acids 1–253 are sufficient for antagonism of L-WNK1 inhibition of ROMK-mediated K⁺ secretion, cannot exclude the possibility that full-length KS-WNK1 may have a different effect in vivo. Finally, KS-WNK1 may also regulate other K⁺ transporters besides ROMK.

Studies using Xenopus oocytes and cultured cells have also suggested that KS-WNK1 may regulate renal Na⁺ transport (27, 28). In the present study, we found that steady-state serum Na⁺ and urinary excretion of Na⁺ are not different between mice overexpressing TG(KS-WNK1(1–253) and wild type. These results, however, do not exclude the physiological role of KS-WNK1 in the regulation of Na⁺ transport in vivo. Because of compensatory responses, steady-state serum and urinary Na⁺ measurement may not reflect renal tubular Na⁺ transport ability. Moreover, amino acids of KS-WNK1 involved in regulation of Na⁺ transporters may reside outside the region of amino acids 1–253. Our results, nevertheless, support the idea that the effect of KS-WNK1(1–253) on K⁺ transport is not secondary to increased urinary excretion of Na⁺.

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