

mTOR Complex-2 Activates ENaC by Phosphorylating SGK1

Ming Lu,* Jian Wang,* Kevin T. Jones,^{†‡} Harlan E. Ives,* Morris E. Feldman,^{§||} Li-jun Yao,* Kevan M. Shokat,^{§||} Kaveh Ashrafi,^{†‡} and David Pearce*

Departments of *Medicine, [†]Physiology, and [§]Cellular and Molecular Pharmacology and [‡]Diabetes Center and ^{||}Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, California

ABSTRACT

The serum- and glucocorticoid-induced kinase 1 (SGK1) plays a central role in hormone regulation of epithelial sodium (Na⁺) channel (ENaC)-dependent Na⁺ transport in the distal nephron. Phosphorylation within a carboxy-terminal domain, designated the hydrophobic motif (HM), determines the activity of SGK1, but the identity of the HM kinase is unknown. Here, we show that the highly conserved serine-threonine kinase mammalian target of rapamycin (mTOR) is essential for the phosphorylation of the HM of SGK1 and the activation of ENaC. We observed that mTOR, in conjunction with rictor (mTORC2), phosphorylated SGK1 and stimulated ENaC. In contrast, when mTOR assembled with raptor in the rapamycin-inhibited complex (mTORC1), it did not phosphorylate SGK1 or stimulate ENaC. Inhibition of mTOR blocked both SGK1 phosphorylation and ENaC-mediated Na⁺ transport, whereas specific inhibition of mTORC1 had no effect. Similarly, small hairpin RNA-mediated knockdown of rictor inhibited SGK1 phosphorylation and Na⁺ current, whereas knockdown of raptor had no effect. Finally, in co-immunoprecipitation experiments, SGK1 interacted selectively with rictor but not with raptor, suggesting selective recruitment of SGK1 to mTORC2. We conclude that mTOR, specifically mTORC2, is the HM kinase for SGK1 and is required for ENaC-mediated Na⁺ transport, thereby extending our understanding of the molecular mechanisms underlying Na⁺ balance.

J Am Soc Nephrol 21: 811–818, 2010. doi: 10.1681/ASN.2009111168

The mammalian target of rapamycin (mTOR) is a highly conserved serine-threonine kinase that integrates multiple inputs, including nutrient abundance and hormonal signals, to orchestrate a variety of cellular processes, including growth, proliferation, and survival.^{1–3} As such, this pathway has been intensely investigated for understanding fundamental aspects of cellular physiology, immune cell function, and development of therapeutic targets for a broad range of disease states.^{1,4} mTOR is organized into two distinct complexes, mTORC1 and mTORC2,^{5–7} which have distinct targets and control distinct cellular processes. mTORC1 consists of mTOR, raptor, PRAS40, and mLST8, whereas mTORC2 contains mTOR, rictor, mSIN1, and mLST8. mTORC1 is central to the control of cell growth through its well-characterized effects on protein synthesis and cell-cycle progression, which are due in part to hydrophobic motif (HM) phosphorylation of the AGC kinase p70-S6K and the oncogene 4E-BP1.^{8,9} Elucidation of mechanisms of

action of mTORC1 has been greatly aided by availability of rapamycin, a small molecule that acutely inhibits mTORC1.^{10,11} Rapamycin has also been widely used clinically as an immune suppressant and chemotherapeutic agent.¹² By contrast, mTORC2 molecular targets and physiologic functions have been less well characterized,³ and, in part due to a lack of specific inhibitors, unambiguous assignment of physiologic functions to this complex has been difficult.¹³

A breakthrough in understanding mechanisms of action of mTORC2 emerged from its identification as

Received November 18, 2009. Accepted January 17, 2010.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. David Pearce, Department of Medicine, University of California, San Francisco, 600 16th Street, San Francisco, CA 94107. Phone: 415-476-7015; Fax: 415-502-3644; E-mail: david.pearce@ucsf.edu

Copyright © 2010 by the American Society of Nephrology

the kinase responsible for phosphorylation of a serine residue at position 473 (S473) within the HM of mammalian Akt, a key survival kinase.¹⁴ The HM of Akt is homologous to that found in other AGC family kinases, including SGK1, raising the possibility that mTORC2 may mediate phosphorylation of the serum- and glucocorticoid-induced kinase 1 (SGK1) HM as well.

In mammals, SGK1 plays a fundamental role in ion and solute transport processes in epithelia.¹⁵ SGK1 is essential for normal sodium (Na⁺) and potassium homeostasis in mice¹⁶ and for Na⁺ transport in cultured cells.¹⁷ A central function of SGK1 is to increase the cell surface expression of the epithelial sodium channel by inhibiting the ubiquitin ligase Nedd4-2.^{18,19} Activation of SGK1 is dependent on phosphorylation of S422 within its HM domain^{20,21}; however, despite considerable progress in understanding the molecular mechanisms underlying SGK1 regulation of solute and ion transport,²² the signaling mechanisms involved in controlling SGK1 activation through HM phosphorylation have remained uncertain. In particular, the HM kinase has remained unknown. In yeast and *Caenorhabditis elegans*, genetic evidence supports the idea that TOR—in particular, TORC2—is the key HM kinase for SGK1 homologues in lower eukaryotes.^{23–25} In mammalian cells, data have been conflicting: On the basis of the effects of small hairpin RNA (shRNA)-mediated knockdown of raptor and rictor, as well as rapamycin inhibition, Hong *et al.*²⁶ concluded that mTORC1 but not mTORC2 binds and activates SGK1 in melanoma cells. Garcia-Martinez and Alessi,²⁷ conversely, found that SGK1 HM phosphorylation and activation were abrogated in mouse embryo fibroblasts deleted of the rictor gene, suggesting a role for mTORC2. Importantly, neither of these studies examined the effects of mTOR on SGK1 within the cellular context for which it has been best characterized, namely regulation of epithelial Na⁺ channel (ENaC).

To determine whether mTOR is the essential HM kinase for SGK1 in kidney tubule cells and, if so, which mTOR complex controls SGK1 activation of ENaC, we used recently developed chemical inhibitors of mTOR⁴ in conjunction with shRNA-mediated knockdown of specific components of mTORC1 or mTORC2 to assign functions unambiguously to each of these complexes. We found that SGK1 HM phosphorylation and SGK1-stimulated Na⁺ transport are dependent on mTORC2-mediated HM phosphorylation in kidney epithelial cells. Furthermore, in co-immunoprecipitation experiments, we found that SGK1 interacts selectively with the mTOR–rictor complex (mTORC2), not with mTORC1. Together, these findings strongly support the idea that mTORC2 is the essential SGK1 HM kinase and is essential for the physiologic control of ENaC-mediated Na⁺ transport.

RESULTS

mTORC2 Controls Phosphorylation of SGK1

By taking advantage of a recently developed, highly selective, ATP-competitive inhibitor of mTOR,⁴ we sought first to determine whether SGK1 HM phosphorylation is acutely depen-

dent on mTOR activity. Unlike rapamycin, this compound, PP242, binds to the active site of mTOR, irrespective of whether it is associated with components of complex 1 or 2, and specifically inhibits outputs of both. PP242 is highly specific in that inhibition occurs at concentrations that do not inhibit 219 other kinases.⁴ We first asked whether PP242 affects SGK1 phosphorylation in mpkCCD cells, a cell line that is derived from the kidney's cortical collecting duct and retains the molecular machinery required for hormone-regulated transepithelial Na⁺ transport.²⁸ In these cells, SGK1 activity is stimulated by insulin through effects on its phosphorylation, and its expression is markedly increased by aldosterone through effects on SGK1 gene transcription.²⁹ We initially used a highly sensitive and specific holo-SGK1 antibody that recognizes both the phosphorylated and unphosphorylated forms of SGK1 to detect phosphorylation of endogenous SGK1 by mobility shift, as described previously.^{21,29–31} In the presence of aldosterone and insulin, we detected multiple SGK1 bands (Figure 1A). The uppermost bands were eliminated by treatment of extracts with λ phosphatase, consistent with previous evidence^{20,29} that they represent phosphorylated SGK1 (Figure 1B). These bands were also eliminated by treatment of the cells with LY294002 (LY; which inhibits all isoforms of phosphatidylinositol-3 kinase, as well as both mTORC1 and mTORC2; Figure 1A), consistent with previous results^{21,29–31} and further supporting the conclusion that the upper bands represent phosphorylated forms of SGK1. We next examined the effect of mTOR inhibition on SGK1 phosphorylation using PP242 (Figure 1A). We found that at 0.3 μM, a concentration that does not inhibit the other relevant kinases (notably phosphatidylinositol-3 kinase, PDK1, Akt, and SGK1 itself), PP242 markedly and rapidly blunted the appearance of these phospho-SGK1 bands. In contrast, rapamycin, which inhibits mTORC1 but not mTORC2, had no effect on SGK1 phosphorylation at concentrations that eliminated phospho p70-S6K (Figure 1A). Furthermore, consistent with previous results,⁴ PP242 but not rapamycin inhibited HM Akt phosphorylation (S473), as detected by both a specific anti-phospho-S473 antibody and a generic anti-phospho-HM antibody (Figure 1A). Akt phosphorylation in the activation loop (T308) also was markedly blunted by PP242 but not by rapamycin (Figure 1A), as previously shown.⁴ This last effect has been shown to be indirect and due to phospho-HM-dependent enhancement of T308 phosphorylation.¹⁴

To look directly at SGK1 S422 phosphorylation, we attempted to establish conditions in which a commercially available antibody (anti-p-SGK1 [S422], Santa Cruz Biotechnology) would specifically recognize endogenous phospho-S422-SGK1; however, in whole-cell lysates, this antibody detected a rapamycin-inhibited species that comigrated at 70 kD precisely with the phosphorylated form of p70-S6K, as detected by a well-characterized antibody (Cell Signaling),⁴ and was distinct from any of the SGK1 species (data not shown). This result is consistent with that of Garcia-Martinez and Alessi²⁷ but not with that of Hong *et al.*²⁶

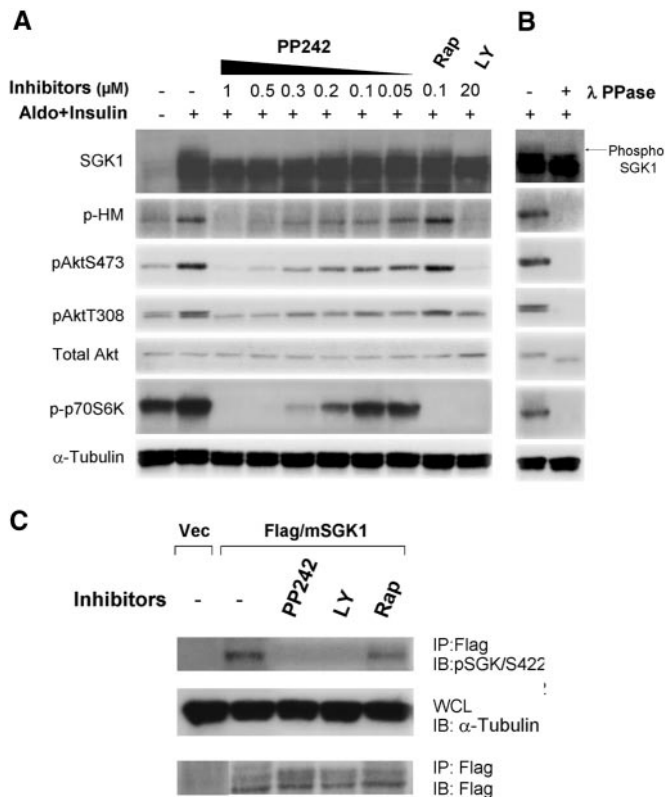


Figure 1. Rapamycin-resistant SGK1 phosphorylation is modulated by mTOR. (A) mpkCCD cells were grown on collagen-coated Transwell polycarbonate membranes and treated with aldosterone and insulin for 4 hours, followed by treatment with inhibitors as shown for 1 hour. Lysates were prepared and analyzed by immunoblot using antibodies against holo-SGK1, phospho-HM, Akt phospho-S473, and phospho-p70S6K, as shown. α -Tubulin is shown as loading control. (B) Phosphatase treatment eliminates mobility-shifted phospho-SGK1 band. mpkCCD cells were grown on Transwell filters and treated with aldosterone and insulin as in A, and whole-cell lysates were treated with λ -phosphatase (λ -PPase) before Western blotting analysis with antibodies, as shown. (C) Immunodetection of HM phosphorylated SGK1 using a commercially available antibody (S422; Santa Cruz Biotechnology). HEK-293 cells were transfected with FLAG-SGK1 or vector control; incubated with insulin for 4 hours; and treated with inhibitors for 1 hour at concentrations of 20 μ M for LY, 0.3 μ M for PP242, and 0.1 μ M for rapamycin. FLAG-SGK1 was enriched by immunoprecipitation with anti-FLAG antibody (see text for details) and immunoblotted as shown.

Although the basis for this discrepancy is uncertain, it is clear that under the present conditions, this antibody does not give a specific signal for endogenous phospho-S422-SGK1. Conversely, when SGK1 was heterologously expressed in HEK-293 cells as a FLAG-fusion protein and its concentration enriched by immunoprecipitation, this antibody did detect a species consistent with phospho-S422-SGK1: It depended on the presence of FLAG-SGK1 expression vector and co-migrated precisely with the phospho-SGK1 band as detected by mobility shift with either anti-

holo-SGK1 or anti-FLAG antibody and well below the band detected by anti-phospho-p70-S6K (data not shown). Consistent with mobility shift data, this band was abrogated by PP242 and LY but not by rapamycin (Figure 1C). Thus, we conclude that a rapamycin-resistant output of mTOR is required for phosphorylation of SGK1 HM in both HEK-293 and mpkCCD cells.

To investigate further whether the rapamycin-resistant SGK1 HM phosphorylation is mediated by mTORC2, we used shRNA directed at the mTORC2-specific component, rictor, in HEK-293 cells transfected with FLAG-SGK1. Using lentiviral-mediated transduction, we were able to achieve a 67% decrease in rictor expression with rictor-specific shRNA (Figure 2A), whereas control shRNA had no significant effect. Concomitantly, FLAG-SGK1 phospho-S422 was reduced by 55% by rictor shRNA but not by control shRNA (Figure 2B). A similar degree of shRNA-mediated knockdown of raptor (68%; Figure 3A) had no effect on SGK1 HM phosphorylation (Figure 3B).

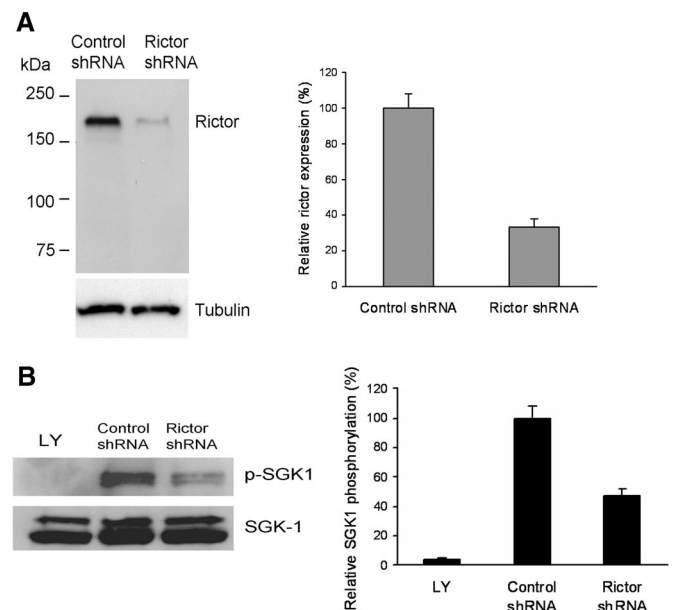


Figure 2. SGK1 phosphorylation is inhibited by knockdown of the expression of rictor. (A) FLAG-SGK1 plasmid was transfected into HEK-293 cells; 24 hours later, cells were infected with recombinant lentiviruses harboring rictor shRNA or control shRNA. After another 24 hours, cells were lysed and analyzed by Western blot using antibodies against rictor; shown is representative blot and quantitative analysis of at least three independent experiments. (B) FLAG-SGK1 was immunoprecipitated from cell lysates in A and analyzed by immunoblot using S422 antibody, as in Figure 1C. The ratio of the signal from anti-p-Sgk1 against anti-holo-Sgk1 was determined and normalized for SGK1 phosphorylation. Shown is a representative blot and quantitative analysis. In each graph, values were significantly different ($P < 0.01$) by unpaired *t* test. Quantification was performed in at least three independent experiments.

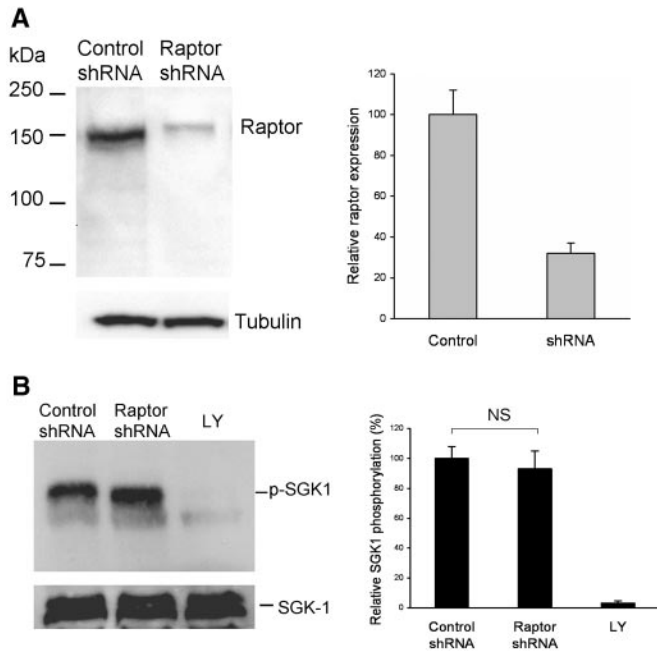


Figure 3. Raptor knockdown has no effect on SGK1 phosphorylation. (A) FLAG-SGK1 plasmid was transfected into HEK-293 cells; after 24 hours, cells were infected with recombinant lentiviruses harboring raptor or control shRNA. After another 24 hours, cells were lysed and analyzed by Western blotting using antibodies against raptor. (B) SGK1 was immunoprecipitated from cell lysates in A and analyzed for HM phosphorylation. The ratio of the signal from anti-p-Sgk1 against anti-holo-Sgk1 was determined and normalized for SGK1 phosphorylation. In each graph, values were significantly different ($P < 0.01$) by unpaired *t* test, except where shown as NS. Quantification was performed in at least three independent experiments.

mTORC2 but not mTORC1 Is Required for Na⁺ Transport in Kidney Epithelial Cells

The best characterized function of SGK1 in mammals is to stimulate ENaC-dependent Na⁺ transport in kidney tubule cells.^{16,17,32,33} To determine whether mTOR activity is important for Na⁺ transport, we examined the effect of PP242 on ENaC-dependent Na⁺ currents in mpkCCD cells grown on Transwell filters. As shown in Figure 4, PP242 completely blocked aldosterone-induced current with an IC₅₀ of approximately 0.2 μM, whereas rapamycin, at concentrations that fully blocked mTORC1, had no effect (Figure 4A). The effect of PP242, like that of LY, was rapid (half-life of approximately 15 minutes) and reversible and occurred without any significant drop in electrical resistance, a sign of tight junction integrity and cell health (Figure 4A). It should be noted that prolonged rapamycin treatment (>16 hours) does inhibit Na⁺ current, an effect that has been attributed to blockade of mineralocorticoid receptor function.³⁴ It also should be noted that prolonged treatment with PP242 or LY (>24 hours) diminishes electrical resistance and causes morphologic changes (*e.g.*, blebbing) in cells, consistent with a toxic effect (J.W., unpublished data, 2009).

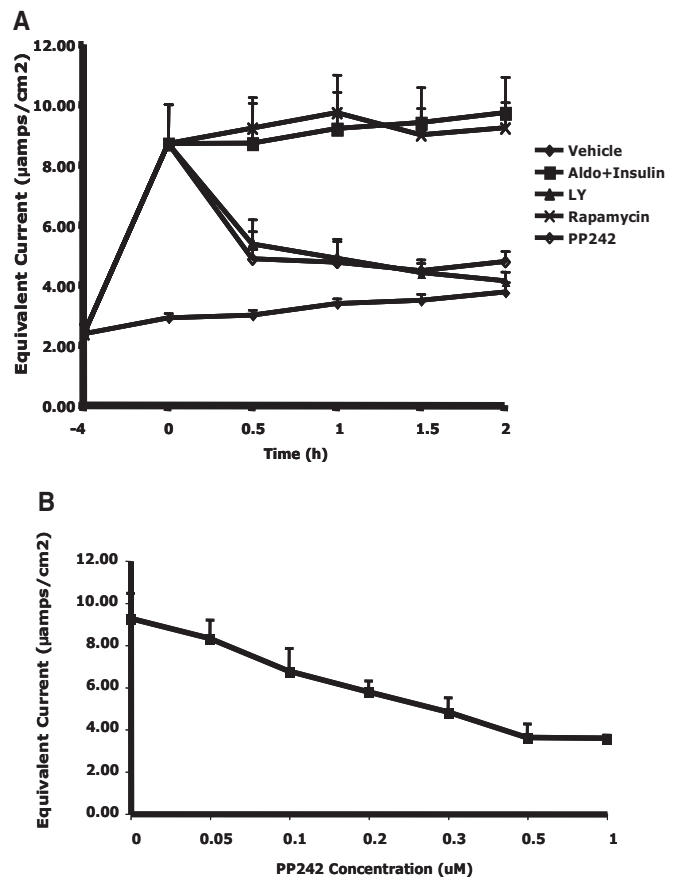


Figure 4. Rapamycin-resistant ENaC-dependent Na⁺ current is modulated by mTOR. (A) mpkCCD cells were grown on Transwell filters, incubated with aldosterone and insulin for 4 hours, and treated with inhibitors for 1 hour at concentrations of 20 μM for LY, 0.3 μM for PP242, and 0.1 μM for rapamycin. Amiloride-sensitive equivalent current was measured by mini-volt-ohm meter (see the Concise Methods section). (B) Concentration dependence of Na⁺ current inhibition by PP242. Cells were treated with aldosterone and insulin as in A and incubated for 2 hours with concentrations of PP242 shown, and equivalent current was determined.

To examine whether knockdown of rictor or raptor expression affects ENaC-dependent Na⁺ transport, we infected mpkCCD cells with the lentiviruses harboring either rictor or raptor shRNA, seeded the cells on Transwell filters, and determined aldosterone-induced Na⁺ currents. Rictor shRNA reduced Na⁺ current by approximately 60% (Figure 5A), which corresponded well to the degree of knockdown in rictor expression (approximately 72%; Figure 5B). In contrast, Na⁺ current was unaffected by a comparable degree of raptor knockdown (Figure 6). Because S6K and 4E-BP1 are widely known substrates for mTORC1, we examined the effects of knockdown of raptor expression on the phosphorylation of S6K and 4E-BP1. Reduction in raptor expression resulted in decreases in S6K and 4E-BP1 phosphorylation in mpkCCD cells (Figure 6C), demonstrating the effectiveness of raptor shRNA in disrupting the function of mTORC1. Together,

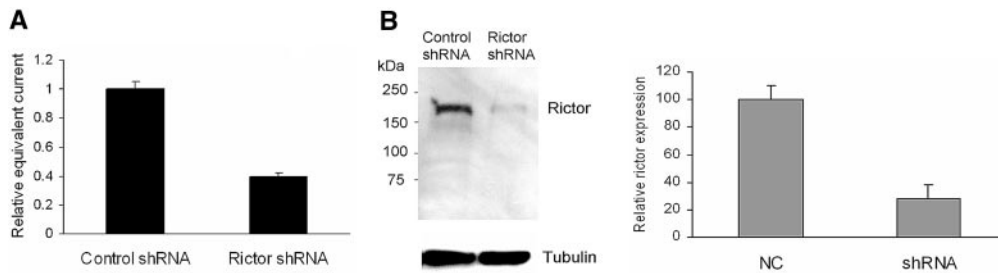


Figure 5. ENaC-dependent Na^+ current is inhibited by knockdown of rictor expression. (A) mpkCCD cells were infected with recombinant lentiviruses harboring rictor shRNA or control shRNA. Infected cells were plated on Transwell filters, and, after reaching high electrical resistance (24 to 72 hours), amiloride-sensitive ENaC-dependent Na^+ currents were measured from 12 Transwell filters for each group in three independent experiments. (B) Infected cells were lysed, and rictor level was determined by Western blot. Values in bar graphs are significantly different ($P < 0.01$) by unpaired *t* test in at least three independent experiments.

these data provide strong support for the conclusion that mTORC2 but not mTORC1 is required for SGK1 HM phosphorylation and ENaC-dependent Na^+ transport.

SGK1 Physically Associates with mTORC2

As a first step toward determining the mechanism underlying the mTORC2-specific regulation of SGK1 HM phosphorylation, we examined SGK1 physical interactions with endogenous mTOR complexes using FLAG-SGK1 transfected into HEK-293 cells. Cells were lysed and FLAG-SGK1 was immunoprecipitated using anti-FLAG antibodies, and immunoprecipitates were analyzed by Western blot using antibodies against mTOR, rictor, and raptor (Figure 7). Strong signals were detected for mTOR and rictor in the FLAG-SGK1 immunoprecipitates, whereas only a faint raptor band was detected (<10% of the intensity of rictor). These data strongly support the conclusion that the majority of SGK1 in the mTOR complexes is associated with mTORC2 and only a minor portion of SGK1 binds to mTORC1.

DISCUSSION

SGK1 is a key mediator of hormone-regulated Na^+ transport in mammalian epithelia.^{16,17,21,32} It has also been suggested to play an important role in cell proliferation and apoptosis^{35,36}; however, the physiologic significance of these latter effects is less certain. Two recent reports found that mTOR is required for SGK1 HM phosphorylation^{27,26}; however, they came to opposite conclusions in regard to which mTOR complex mediates SGK1 phosphorylation. In this report, we used both chemical inhibitors and shRNA-mediated knockdown to show that mTORC2 but not mTORC1 is essential for phosphorylation of SGK1 and activation of ENaC-mediated Na^+ transport. Using a specific inhibitor of mTOR catalytic activity, PP242, which blocks both mTORC1- and mTORC2-dependent outputs, we observed a marked rapid reduction in SGK1 phosphorylation (Figure 1) and in ENaC-dependent Na^+ current (Figure 4). The mTORC1-specific inhibitor, rapamycin, had minimal effect on either SGK1 phosphorylation or Na^+ current (Figures 1

and 4). To determine independently whether mTORC1 and/or mTORC2 is required for SGK1 phosphorylation and physiologic function, we used shRNA-mediated knockdown of raptor and rictor, which are essential components of mTORC1 and mTORC2, respectively. Consistent with the chemical inhibitor data, shRNA-mediated knockdown of rictor resulted in a significant decrease in SGK1 HM phosphorylation (Figure 2) and ENaC-dependent Na^+ transport (Figure 5), whereas knockdown of raptor had no effect. Both rapamycin and raptor knockdown were shown to inhibit mTORC1 effectively, because phosphorylation of two known mTORC1 substrates, S6K and 4E-BP1, was blocked (Figure 6). On the basis of coimmunoprecipitation experiments, it seems that the selective regulation of SGK1 by mTORC2 is based on its selective physical interaction with components of this complex, likely rictor or possibly Sin1 (Figure 7). Further work is needed to determine the mechanistic basis for the association between SGK1 and mTORC2 and, in particular, which components of mTORC2 are responsible for the selective physical interaction.

Our study has revealed a novel role for mTORC2 in SGK1-dependent regulation of ENaC-dependent Na^+ transport and further supports the idea that the mTOR complexes are important regulators not only of cell growth and proliferation but also of cellular homeostatic activities as diverse as actin cytoskeleton integrity⁷ and Na^+ transport. Our findings are consistent with those of Garcia-Martinez and Alessi²⁷ in murine embryo fibroblasts, as well as findings in *Saccharomyces cerevisiae*²⁴ and *C. elegans*,^{23,25} all of which identified TORC2 as the SGK1 HM kinase. Conversely, our findings are not consistent with those of Hong *et al.*²⁶ in a melanoma cell line. It is possible that the distinct mTOR complexes phosphorylate SGK1 in different cellular contexts or that the discrepancies are due to technical issues. In our hands, the commercially available antiserum, raised against SGK1 HM peptide (Santa Cruz Biotechnology), was not specific when used in whole-cell lysates and in fact detected phospho-S6K—the prototypical substrate of mTORC1—but not SGK1 (data not shown). Detection of phospho-SGK1 required enrichment through immunoprecipitation. Garcia-Martinez and Alessi²⁷ reported a similar experience with this antibody.

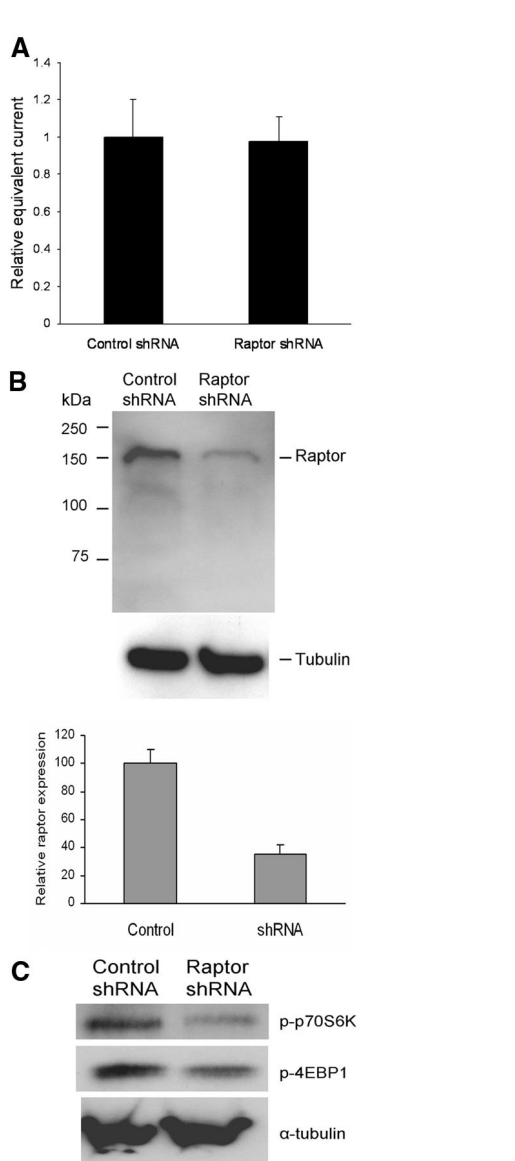


Figure 6. Raptor knockdown has no effect on ENaC-dependent Na⁺ current. (A) mpkCCD cells were infected with recombinant lentiviruses harboring raptor or control shRNA and plated on Transwell filters, and amiloride-sensitive ENaC-dependent Na⁺ currents were measured, as in Figure 5. (B) Infected cells were lysed, and raptor level was determined by Western blot. Values in bar graphs are significantly different ($P < 0.01$) by unpaired t test in at least three independent experiments. (C) Infected cells were lysed and probed by Western blot using antibodies against the raptor substrates p-p70S6K and p-4EBP1, respectively.

In summary, our findings suggest a mechanistic basis for the activation of SGK1 in the control of ENaC-mediated Na⁺ transport (shown schematically in Figure 8). According to this view, mTORC2, which is activated by insulin and IGF-1 through an as-yet-undetermined mechanism, physically associates with SGK1 and phosphorylates its HM (S422). The phosphorylated HM then provides a docking site for PDK1, which phosphorylates T256 within the activation loop³⁷; the

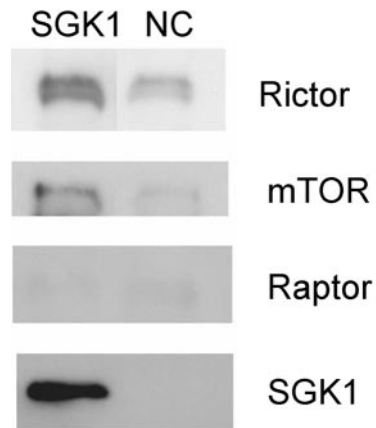


Figure 7. SGK1 associates preferentially with mTORC2. A plasmid harboring FLAG-SGK1 was transfected into HEK-293 cells. A GFP-containing plasmid was used as negative control (NC). Forty-eight hours after transfection, the cells were lysed and immunoprecipitated for SGK1 with anti-FLAG antibody. Bound proteins were recovered and analyzed by Western blot using antibodies against rictor, mTOR, raptor, or the FLAG tag, respectively.

fully activated kinase is recruited by GILZ1 to substrates that are themselves associated with ENaC.³⁸ These substrates, including Nedd4-2 and cRaf, are phosphorylated and inhibited, and thus ENaC residence at the plasma membrane is increased.^{19,38,39} Through this mechanism, SGK1 activation and physiologic effects are selectively controlled by mTORC2 in the absence of mTORC1-dependent changes in protein synthesis and cellular proliferation. These data provide further insight into the molecular mechanism(s) underlying Na⁺ balance and BP regulation and suggest a mechanism for the regulation of a specific physiologic process through selective recruitment of pleiotropic signaling molecules. Specific inhibitors of mTORC2 may be useful in the treatment of disorders of tubular transport, including salt-sensitive hypertension and congestive heart failure.

CONCISE METHODS

Generation of Recombinant Lentiviruses Harboring Rictor or Raptor shRNAs

Synthesized sense and antisense oligos representing shRNAs for raptor or rictor were annealed using a touchdown protocol on a PTC-200 thermal cycler at 95°C for 30 seconds and 60°C for 10 minutes, then cooled to 20°C at 1°C every 15 seconds. The annealed shRNAs were ligated with the pLentiLox 3.7 vector digested with XhoI/HpaI and treated with calf intestinal alkaline phosphatase. The ligated DNA was transformed into DH5 α -competent bacterial cells. Ampicillin-resistant colonies were picked and grown in LB broth for 16 hours. Plasmid DNA was isolated using mini-prep columns (Fermentas). Positive recombinants were identified by restriction enzyme digestion and verified by DNA sequencing. Recombinant lentiviruses were generated by co-transfection of plasmids harboring the shRNAs and a mixture of packaging plasmids into HEK-293T packaging cells. Viral

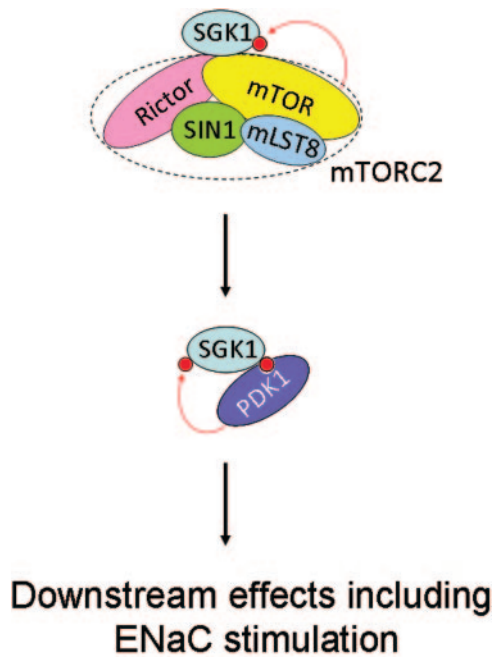


Figure 8. SGK1 activation is mediated by mTORC2 in the regulation of ENaC. SGK1 associates with mTORC2 (delineated by dashed oval), which phosphorylates S422 in the HM. HM phosphorylated SGK1 interacts with PDK1,³⁷ which phosphorylates T256 (small red circles represent phosphate groups, and red arrows represent phosphorylation events).

supernatants were harvested 48 hours after transfection. For determination of the viral titer, a 10-fold dilution series of viruses was made and used to infect fresh HEK-293T cells. A viral titer of 5×10^5 /ml was routinely observed by visualizing cells for EGFP fluorescence.

Cell Culture and Recombinant DNA Transduction

HEK-293 cells were regularly maintained in plastic tissue culture flasks at 37°C in DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. Cells were seeded on 10-cm dishes (3×10^6 cells/dish) and allowed to grow overnight. They were then transfected with 5 μ g of pMO/Flag/mSGK1 (Flag-epitope at N-terminal of mouse SGK1) or the empty vector using lipofectamine according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells were maintained in serum-free DMEM supplemented with 10% Hyclone Cell Boost 1 supplement (Hyclone, Logan, UT) for 24 hours before treatment with 100 nM insulin for 1 hour and then treated with 0.3 μ M PP242, 20 μ M LY, or 0.1 μ M rapamycin for 1 hour.

Renal epithelial cells, mpkCCDc14, were maintained in plastic tissue culture flasks in modified DMEM/Ham's F12 (1:1) medium ("Regular medium") as described previously.²⁹ Lentivirus-mediated gene transduction was carried out at a multiplicity of infection of 2.

Immunoprecipitation and Immunoblotting

Transfected or lentivirus-infected cells were lysed in binding buffer (50 mM Tris-HCl [pH 7.5], 10% glycerol, 1 mM EDTA, 2 mM dithiothreitol, 150 mM NaCl, 1% Triton X-100, or 0.3% CHAPS) for 15 minutes. After centrifugation, the supernatants were collected and incubated with the anti-flag M2 affinity beads (Sigma, St. Louis, MO). The immunoprecipi-

tates were collected by centrifugation, washed three times, and boiled for 5 minutes in 50 μ l of cracking buffer (50 mM Tris-HCl [pH 7.0], 10% glycerol, 2% SDS, and 2% β -mercaptoethanol). Immunoblotting was carried out by separating the immunoprecipitates on 10% polyacrylamide gels as described using a Bio-Rad minigel apparatus and transferred electrophoretically to Hybond-C Extra membranes (GE Healthcare) using a Trans-Blot apparatus (Bio-Rad). The membranes were incubated to block nonspecific binding in 5% nonfat dry milk in T-PBS (1.5 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 2.7 mM KCl, 130 mM NaCl, and 0.1% Tween 20) with gentle agitation for 1 hour at room temperature and probed by Western blotting (for endogenous and transfected proteins, as described in figure legends), using antibodies against rictor, raptor, mTOR, Akt phospho-S473, Akt phospho-T308, phospho-p70-S6K, and pan-phospho-HM (Cell Signaling); anti- α -tubulin (Calbiochem); anti-phospho-SGK1 (S422; Santa Cruz Biotechnology); and anti-SGK1 (a gift from Gary Firestone, University of California Berkeley). After washing with T-PBS, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG in T-PBS for 1 hour, washed three times in T-PBS, and incubated with ECL Plus Western Blotting Detection System working solution (GE Healthcare) according to the manufacturer's instructions.

Measurement of ENaC-Dependent Na^+ Transport

For electrophysiologic measurements, mpkCCDc14 cells were seeded on type VI collagen (Sigma) coated filters (Transwell, pore-size 0.4 μ m; Corning Costar) and grown at least 24 hours before treatment with aldosterone at a concentration of 1 μ M in the presence and absence of PP242, LY, and rapamycin. Transepithelial resistance and potential difference across the cell monolayer were measured using a mini-volt-ohm meter (MilliCell ERS; Millipore) at specified time points after treatment. The equivalent short-circuit current was calculated using Ohm's law.

ACKNOWLEDGEMENTS

Gary Firestone is gratefully acknowledged for providing SGK1 antibody, Joyce Slingerland for providing WM35 cells, and Alain Vandewalle for providing mpkCCD cells. We thank Holly Ingraham and Rama Soundararajan for helpful comments on the manuscript.

DISCLOSURES

None.

REFERENCES

1. Wullschlegel S, Loewith R, Hall MN: TOR signaling in growth and metabolism. *Cell* 124: 471–484, 2006
2. Guertin DA, Sabatini DM: Defining the role of mTOR in cancer. *Cancer Cell* 12: 9–22, 2007
3. Huang J, Manning BD: A complex interplay between Akt, TSC2 and the two mTOR complexes. *Biochem Soc Trans* 37: 217–222, 2009
4. Feldman ME, Apsel B, Uotila A, Loewith R, Knight ZA, Ruggero D,

- Shokat KM: Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. *PLoS Biol* 7: e38, 2009
5. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K: Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* 110: 177–189, 2002
 6. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM: mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* 110: 163–175, 2002
 7. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM: Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* 14: 1296–1302, 2004
 8. Fingar DC, Blenis J: Target of rapamycin (TOR): An integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* 23: 3151–3171, 2004
 9. Hay N, Sonenberg N: Upstream and downstream of mTOR. *Genes Dev* 18: 1926–1945, 2004
 10. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, Schreiber SL: A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* 369: 756–758, 1994
 11. Kunz J, Henriquez R, Schneider U, Deuter-Reinhard M, Movva NR, Hall MN: Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell* 73: 585–596, 1993
 12. Yocum DE: Cyclosporine, FK-506, rapamycin, and other immunomodulators. *Rheum Dis Clin North Am* 22: 133–154, 1996
 13. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM: Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* 22: 159–168, 2006
 14. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM: Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307: 1098–1101, 2005
 15. Lang F, Bohmer C, Palmada M, Seeböhm G, Strutz-Seeböhm N, Vallon V: (Patho)physiological significance of the serum- and glucocorticoid-inducible kinase isoforms. *Physiol Rev* 86: 1151–1178, 2006
 16. Wulff P, Vallon V, Huang DY, Volkl H, Yu F, Richter K, Jansen M, Schlunz M, Klingel K, Loffing J, Kauselmann G, Bosl MR, Lang F, Kuhl D: Impaired renal Na⁺ retention in the sgk1-knockout mouse. *J Clin Invest* 110: 1263–1268, 2002
 17. Chen SY, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, Firestone GL, Verrey F, Pearce D: Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc Natl Acad Sci U S A* 96: 2514–2519, 1999
 18. Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraïbi A, Pratt JH, Horisberger JD, Pearce D, Loffing J, Staub O: Phosphorylation of Nedd4–2 by Sgk1 regulates epithelial Na⁺ channel cell surface expression. *EMBO J* 20: 7052–7059, 2001
 19. Snyder PM, Olson DR, Thomas BC: Serum and glucocorticoid-regulated kinase modulates Nedd4–2-mediated inhibition of the epithelial Na⁺ channel. *J Biol Chem* 277: 5–8, 2002
 20. Park J, Leong ML, Buse P, Maiyar AC, Firestone GL, Hemmings BA: Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. *EMBO J* 18: 3024–3033, 1999
 21. Webster MK, Goya L, Ge Y, Maiyar AC, Firestone GL: Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Mol Cell Biol* 13: 2031–2040, 1993
 22. Bhalla V, Soundararajan R, Pao AC, Li H, Pearce D: Disinhibitory pathways for control of sodium transport: Regulation of ENaC by SGK1 and GILZ. *Am J Physiol Renal Physiol* 291: F714–F721, 2006
 23. Jones KT, Greer ER, Pearce D, Ashrafi K: Rictor/TORC2 regulates *Caenorhabditis elegans* fat storage, body size, and development through sgk-1. *PLoS Biol* 7: e60, 2009
 24. Kamada Y, Fujioka Y, Suzuki NN, Inagaki F, Wullschlegel S, Loewith R, Hall MN, Ohsumi Y: Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization. *Mol Cell Biol* 25: 7239–7248, 2005
 25. Soukas AA, Kane EA, Carr CE, Melo JA, Ruvkun G: Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in *Caenorhabditis elegans*. *Genes Dev* 23: 496–511, 2009
 26. Hong F, Larrea MD, Doughty C, Kwiatkowski DJ, Squillace R, Slingerland JM: mTOR-raptor binds and activates SGK1 to regulate p27 phosphorylation. *Mol Cell* 30: 701–711, 2008
 27. Garcia-Martinez JM, Alessi DR: mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochem J* 416: 375–385, 2008
 28. Bens M, Vallet V, Cluzeaud F, Pascual-Letallec L, Kahn A, Rafestain-Oblin ME, Rossier BC, Vandewalle A: Corticosteroid-dependent sodium transport in a novel immortalized mouse collecting duct principal cell line. *J Am Soc Nephrol* 10: 923–934, 1999
 29. Wang J, Knight ZA, Fiedler D, Williams O, Shokat KM, Pearce D: Activity of the p110-alpha subunit of phosphatidylinositol-3-kinase is required for activation of epithelial sodium transport. *Am J Physiol Renal Physiol* 295: F843–F850, 2008
 30. Loffing J, Zecevic M, Feraille E, Kaissling B, Asher C, Rossier BC, Firestone GL, Pearce D, Verrey F: Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: Possible role of SGK. *Am J Physiol Renal Physiol* 280: F675–F682, 2001
 31. Wang J, Barbry P, Maiyar AC, Rozansky DJ, Bhargava A, Leong M, Firestone GL, Pearce D: SGK integrates insulin and mineralocorticoid regulation of epithelial sodium transport. *Am J Physiol Renal Physiol* 280: F303–F313, 2001
 32. Loffing J, Flores SY, Staub O: Sgk kinases and their role in epithelial transport. *Annu Rev Physiol* 68: 461–490, 2006
 33. Naray-Fejes-Toth A, Canessa C, Cleaveland ES, Aldrich G, Fejes-Toth G: Sgk is an aldosterone-induced kinase in the renal collecting duct: Effects on epithelial Na⁺ channels. *J Biol Chem* 274: 16973–16978, 1999
 34. Edinger RS, Watkins SC, Pearce D, Johnson JP: Effect of immunosuppressive agents on glucocorticoid receptor function in A6 cells. *Am J Physiol Renal Physiol* 283: F254–F261, 2002
 35. Brunet A, Park J, Tran H, Hu LS, Hemmings BA, Greenberg ME: Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol* 21: 952–965, 2001
 36. Mikosz CA, Brickley DR, Sharkey MS, Moran TW, Conzen SD: Glucocorticoid receptor-mediated protection from apoptosis is associated with induction of the serine/threonine survival kinase gene, sgk-1. *J Biol Chem* 276: 16649–16654, 2001
 37. Biondi RM, Kieloch A, Currie RA, Deak M, Alessi DR: The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J* 20: 4380–4390, 2001
 38. Soundararajan R, Melters D, Shih IC, Wang J, Pearce D: Epithelial sodium channel regulated by differential composition of a signaling complex. *Proc Natl Acad Sci U S A* 106: 7804–7809, 2009
 39. Kamynina E, Staub O: Concerted action of ENaC, Nedd4–2, and Sgk1 in transepithelial Na⁺ transport. *Am J Physiol Renal Physiol* 283: F377–F387, 2002