mTOR Complex-2 Activates ENaC by Phosphorylating SGK1

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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.


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.¹,²,⁴ 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...
the kinase responsible for phosphorylation of a serine residue at position 473 (S473) within the HM of mammalian Akt, a key survival kinase.\textsuperscript{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.\textsuperscript{15} SGK1 is essential for normal sodium (Na\textsuperscript{+}) and potassium homeostasis in mice\textsuperscript{16} and for Na\textsuperscript{+} transport in cultured cells.\textsuperscript{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.\textsuperscript{18,19} Activation of SGK1 is dependent on phosphorylation of S422 within its HM domain\textsuperscript{20,21}; however, despite considerable progress in understanding the molecular mechanisms underlying SGK1 regulation of solute and ion transport,\textsuperscript{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 \textit{Caenorhabditis elegans}, genetic evidence supports the idea that TOR—in particular, TORC2—is the key HM kinase for SGK1 homologues in lower eukaryotes.\textsuperscript{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 \textit{et al.}\textsuperscript{26} concluded that mTORC1 but not mTORC2 binds and activates SGK1 in melanoma cells. Garcia-Martinez and Alessi,\textsuperscript{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\textsuperscript{+} 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 mTOR\textsuperscript{4} 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\textsuperscript{+} 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\textsuperscript{+} transport.

RESULTS

mTORC2 Controls Phosphorylation of SGK1

By taking advantage of a recently developed, highly selective, ATP-competitive inhibitor of mTOR,\textsuperscript{4} we sought first to determine whether SGK1 HM phosphorylation is acutely dependent 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.\textsuperscript{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\textsuperscript{+} transport.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 A phosphatase, consistent with previous evidence\textsuperscript{20,22} 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\textsuperscript{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 \muM, 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,\textsuperscript{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.\textsuperscript{4} This last effect has been shown to be indirect and due to phospho–HM–dependent enhancement of T308 phosphorylation.\textsuperscript{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),\textsuperscript{4} and was distinct from any of the SGK1 species (data not shown). This result is consistent with that of Garcia-Martinez and Alessi\textsuperscript{27} but not with that of Hong \textit{et al.}\textsuperscript{26}
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).

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three independent experiments. Where shown as NS. Quantification was performed in at least two 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. 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 blocked aldosterone-induced current with an IC50 of approximately 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,
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. It has also been suggested to play an important role in cell proliferation and apoptosis; however, the physiologic significance of these latter effects is less certain. Two recent reports found that mTOR is required for SGK1 HM phosphorylation; 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 co-immunoprecipitation 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 integrity and Na⁺ transport. Our findings are consistent with those of Garcia-Martinez and Alessi in murine embryo fibroblasts, as well as findings in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, all of which identified TORC2 as 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 Alessi reported a similar experience with this antibody.
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 loop; the fully activated kinase is recruited by GILZ1 to substrates that are themselves associated with ENaC. These substrates, including Nedd4-2 and cRaf, are phosphorylated and inhibited, and thus ENaC residence at the plasma membrane is increased. 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 rictor or raptor 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 DH5α competent bacterial cells. Ampicillin-resistant colonies were picked and grown in LB broth for 16 hours. Plasmid DNA was isolated using mini-prep columns. 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
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 $5 \times 10^5$ 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 ($3 \times 10^6$ cells/dish) and allowed to grow overnight. They were then transfected with $5 \mu$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 $\mu$M PP242, 20 $\mu$M LY, or 0.1 $\mu$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. 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 immunoprecipitates were collected 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 $5 \times 10^5$ ml was routinely observed by visualizing cells for EGFP fluorescence.

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 $\mu$m; Corning Costar) and grown at least 24 hours before treatment with aldosterone at a concentration of 1 $\mu$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

4. Feldman ME, Apsel B, Uotila A, Loewirth R, Knight ZA, Ruggiero D,
Shokat KM: Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol 7: e38, 2009