Regulation of a Third Conserved Phosphorylation Site in SGK1*

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SGK1 (serum- and glucocorticoid-induced kinase 1) is a member of the AGC branch of the protein kinase family. Among well described functions of SGK1 is the regulation of epithelial transport through phosphorylation of the ubiquitin protein ligase Nedd4-2 (neuronal precursor cell expressed developmentally down-regulated 4-2). The activation of SGK1 has been widely accepted to be dependent on the phosphorylation of Thr256 in the activation loop and Ser422 in the hydrophobic motif near the C terminus. Here, we report the identification of two additional phosphorylation sites, Ser397 and Ser401. Both are required for maximum SGK1 activity induced by extracellular agents or by coexpression with other protein kinases, with the largest loss of activity from mutation of Ser397. Coexpression with active Akt1 increased the phosphorylation of Ser397 and thereby SGK1 kinase activity. SGK1 activation was further augmented by coexpression with the protein kinase WNK1 (with no lysine kinase 1). These findings reveal further complexity underlying the regulation of SGK1 activity.

SGK1 (serum- and glucocorticoid-induced kinase 1) is a serine/threonine protein kinase originally identified from a differential screen for glucocorticoid-induced transcripts in a mammary tumor cell line (1). It belongs to the AGC branch of the protein kinase family and is related to Akt (also known as protein kinase B), with ~55% identity in the kinase domain (2). SGK1 plays an important role in the regulation of epithelial ion transport (3); SGK1-null mice display a defect in sodium homeostasis due to disturbed renal tubular handling of sodium (4). The epithelial sodium channel (ENaC) is located in the apical membrane of aldosterone-responsive epithelia, including the renal collecting duct. Because the average open probability of ENaC is high, sodium transport depends significantly on the number of channels on the apical surface of epithelial cells. SGK1 phosphorylates the ubiquitin ligase Nedd4-2 (neuronal precursor cell expressed developmentally down-regulated 4-2), which decreases the interaction of Nedd4-2 with ENaC. As a consequence, ubiquitylation of ENaC is reduced, and its internalization decreases. Thus, SGK1 enhances ENaC abundance in the cell membrane and thereby increases sodium reabsorption (3, 5). A number of other transport proteins are also thought to be influenced by SGK1 (6).

Like some other AGC kinases, SGK1 activity is regulated by phosphorylation. After stimulation, SGK1 becomes phosphorylated at Ser422 in a C-terminal region that extends from the core kinase domain, termed the hydrophobic motif. Ser422 can be phosphorylated by mTOR and perhaps DNA-dependent protein kinase and other protein kinases (7, 8). Phosphorylation of Ser422 transforms SGK1 into a substrate for the phosphoinositide-dependent protein kinase PDK1. PDK1 binds to the hydrophobic motif on SGK1, promoting phosphorylation of Thr256 in the SGK1 activation loop and causing its activation (2). This mechanism is similar to that originally described for Akt, aside from the fact that SGK1 has no pleckstrin homology domain.

In certain AGC kinases, a third site, termed the turn motif site, lies at the C terminus of the core kinase domain preceding the hydrophobic motif (9). Mutation of this site significantly reduces phosphorylation of the hydrophobic motif site and kinase activity in protein kinase C and in some other AGC family members (10–14). A comparison of AGC kinases suggests that many have the turn motif site and may utilize this site for regulation of activity (15). The predicted turn motif site in SGK1 has not been reported to affect SGK1 activity.

We found previously that WNK1, a protein kinase overexpressed in a rare form of hypertension, stimulates SGK1 activity by a mechanism independent of WNK1 catalytic activity (16, 17). In contrast, although closely related to SGK1, Akt and p70 S6 kinase do not appear to be regulated by WNK1. Through comparisons of these protein kinases, we found that Akt1 activity is required for SGK1 activation by WNK1 (17). Akt phosphorylates WNK1 at Thr58 (18). Mutation of Thr58 significantly reduces, but does not eliminate, activation of SGK1 by WNK1. In examining this mechanism further, we found that coexpression of WNK1(T58A) with Akt1 increased SGK1 activity, suggesting that phosphorylation of Thr58 may not be the only action of Akt1 in this system. Here, we provide evidence that the turn motif phosphorylation site of SGK1 is critical for its full activity and that phosphorylation of the SGK1 turn motif is dramatically increased by coexpression with Akt1.

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4 The abbreviations used are: ENaC, epithelial sodium channel; GST, glutathione S-transferase; HA, hemagglutinin; IGF1, insulin-like growth factor 1; Myr-Akt1, myristoylated Akt1.
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MATERIALS AND METHODS

Constructs, Proteins, Reagents, and Antibodies—pCMV5-Myc-WNK1(1–491) (wild-type and T58A), pCMV7–3×FLAG-SGK1, pCMV7–3×FLAG-ΔSGK1 (residues 61–428), pCMV7–3×FLAG-ΔSGK1KD (kinase-dead), pcDNA3-Myc-PDK1, pCMV-HA-Akt1, and glutathione S-transferase (GST)-Nedd4-2 were expressed in bacterial strain BLR(DE3)pLys (Novagen) and purified as described (16). Site-directed mutagenesis was performed using the QuikChange kit (Stratagene) and confirmed by sequencing.

The anti-hemagglutinin (HA) antibody (12CA5) was from Berkeley Antibody Co., and the anti-Myc antibody (9E10) was from the National Cell Culture Center. The anti-FLAG monoclonal antibody was from Sigma. The anti-SGK1 phospho-Thr256 and phospho-Ser422 antibodies were from Santa Cruz Biotechnology. Antibodies recognizing WNK1, OSR1, and ERK1/2 were as described (19, 20). The anti-phospho-ERK1/2 antibody was from Cell Signaling Technology. All antibodies were used at a dilution of 1:1000 for immunoblotting and 1:100 for immunoprecipitation. Insulin-like growth factor 1 (IGF1) was from Calbiochem. Other chemicals were from Sigma.

Cell Culture, Transfection, and RNA Interference—HeLa cells and the breast cancer cell line MDA-MB-231 were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and 100 units/ml penicillin/streptomycin at 37 °C under 5% CO2. Cells were transfected with constructs by Lipofectamine 2000 or with small interfering RNA oligonucleotides by Lipofectamine 2000 RNAiMax following the manufacturer’s protocols (Invitrogen). WNK1 and Akt1 RNA interference oligonucleotide sequences were as follows (Ambion): human WNK1.1, cagaccgucgcaaucTT (sense) and guggacuacagcucuTT (antisense); and human Akt1, gacgccuucugcuuugucaTT (sense) and ugaacagagaaggccgTT (antisense).

Preparation of Cell Lysates and Immunoblotting—Cultured cells were homogenized in lysis buffer (50 mM HEPES (pH 7.6), 150 mM sodium chloride, 0.5% Triton X-100, 10% glycerol, 0.5 mM sodium orthovanadate, 10 mM HEPES (pH 7.6), 5 mM ATP (5 μCi of [γ-32P]ATP), 10 mM MgCl2, and 10 mM β-glycerol phosphate) at 30 °C for 45 min.

Identification of Phosphorylation Sites—Overexpressed protein was immunoprecipitated as described above and resolved by SDS-PAGE. Gels were stained with a colloidal blue staining kit following the manufacturer’s protocol (Invitrogen). Protein bands were extracted from the gel and digested with trypsin. Phosphorylation sites were identified by precursor ion scanning and nanoelectrospray tandem mass spectrometry as described (21).

RESULTS

SGK1 Is Activated upon Coexpression with Active Akt1—We showed previously that SGK1 could be activated by a fragment of WNK1 and that activation required the catalytic activity of Akt1 (17). A kinase-dead mutant of Akt1 reduced SGK1 activation by WNK1. To explore how Akt1 is involved in SGK1 activation, wild-type Akt1, a constitutively active mutant, myristoylated Akt1 (Myr-Akt1), and the activation loop kinase PDK1 were coexpressed with truncated SGK1 (ΔSGK1) or its kinase-dead form in different combinations as shown in Fig. 1. Although wild-type Akt1 did not increase SGK1 activity toward its substrate Nedd4-2, Myr-Akt1 did significantly increase SGK1 activity. PDK1 also increased SGK1 activity, but greater SGK1 activity was observed by coexpression with PDK1 plus Akt1. No activation was observed with kinase-dead ΔSGK1, indicating that Nedd4-2 was phosphorylated by SGK1, not by other kinases contaminating the immunoprecipitate. Similar experiments performed with full-length SGK1 revealed less marked activation. The first 60 amino acids are thought to be involved primarily in SGK1 degradation, not activity (22, 23).
Therefore, SGK1 was used for the majority of experiments except as noted.

The findings above suggest that Akt1 kinase activity is involved in SGK1 activation. To determine whether WNK1 is required for SGK1 activation by Akt1, Myr-Akt1 and SGK1 were expressed in cells in which endogenous WNK1 was knocked down using RNA interference. No obvious decrease in SGK1 activation was observed, suggesting that activation of SGK1 by Akt1 is independent of WNK1 (data not shown).

**SGK1 Ser^397 Is Phosphorylated in Cells Coexpressing Myr-Akt1**—To identify possible modifications on SGK1, FLAG-SGK1 was expressed alone or with Myr-Akt1 in HeLa cells. SGK1 was immunoprecipitated with an anti-FLAG monoclonal antibody, and the protein bands were excised from a denaturing gel for mass spectrometry. The analysis revealed that Ser^401 was constitutively phosphorylated in SGK1. In addition to Ser^401, Ser^397 was also phosphorylated in cells coexpressing Myr-Akt1 (Fig. 2A). The SGK1 activation loop and hydrophobic motif sites, Thr^256 and Ser^422, are required for activation. Alignment of SGK1 with other AGC family members, Akt1 and p70 S6 kinase, shows that Ser^397 is conserved and aligns with the turn motif site in these related protein kinases (Fig. 2B), as suggested recently (15). In protein kinase C, this site is critical in stabilizing the kinase fold by anchoring the C terminus at the top of the N-terminal portion of the kinase core (24). Fig. 8 shows a model of the SGK1 structure indicating the position of Ser^397.

To test the importance of Ser^397 and Ser^401 in SGK1 activity, the residues were mutated to Ala (S^397A and S^401A). As shown in Fig. 2C, SGK1(S^397A) had little kinase activity; activity was low despite coexpression with Myr-Akt1, PDK1, or PDK1 plus Akt1. This indicates that Ser^397 is essential for SGK1 activation. SGK1(S^401A) also showed a reduced capacity to be activated, consistent with a role in regulating SGK1 activity.

**Effects of Mutating Phosphorylatable Residues**—As reported previously (25) mutation of the hydrophobic motif site (Ser^422) to Asp increased SGK1 activity and facilitated further activation by PDK1 (Fig. 3A). As expected, there was some reactivity of the S^422D mutant with the anti-SGK1 phospho-Thr^256 antibody, consistent with its increased kinase activity. Interestingly, the S^422D mutant was also recognized by the anti-SGK1 phospho-Ser^222 antibody. Mutation of Ser^401 to Ala did not interfere with these enhancing effects of S^422D. On the other hand, mutation of Ser^397 to Ala prevented the SGK1 activity increase associated with the S^422D mutant; the reduction could be detected not only by kinase assay but also by reduced phospho-
Thr\textsuperscript{256} reactivity. To determine whether acidic substitutions for Ser\textsuperscript{397} or Ser\textsuperscript{401} might positively impact SGK1 activity, these residues were mutated singly and in combination to Asp. S397D showed little enhancement of activity in either truncated or full-length SGK1 and no increase in reactivity with either of the phospho-specific SGK1 antibodies (Fig. 3, A and B). In contrast, truncated SGK1(S401D) showed a modest elevation in activity compared with the wild type, whereas the full-length S401D mutant was even more active than the S422D mutant. The double S397D/S401D mutants showed little activity above the wild type in either truncated or full-length SGK1.

Phosphorylation of Ser\textsuperscript{397} Is Necessary for Stimulation of SGK1 by IGF1 and H\textsubscript{2}O\textsubscript{2}—IGF1 and H\textsubscript{2}O\textsubscript{2}—SGK1 is activated by growth factors and cell stresses, including IGF1 and H\textsubscript{2}O\textsubscript{2} (25, 26). H\textsubscript{2}O\textsubscript{2} activated full-length SGK1 in HeLa cells much more than did IGF1 (Fig. 4B and data not shown). To detect if the phosphorylation of Ser\textsuperscript{397} is necessary for the activation of SGK1 by these two agents, cells expressing truncated or full-length wild-type SGK1 or the S397A or S401A mutant of truncated or full-length SGK1 were treated with IGF1 and H\textsubscript{2}O\textsubscript{2}. Mutation of Ser\textsuperscript{397} to Ala blocked stimulation of SGK1(S397A) and SGK1(S401A) by either IGF1 or H\textsubscript{2}O\textsubscript{2} (Fig. 4, A and B). Results were similar with the double S397A/S401A mutant. In contrast, mutation of Ser\textsuperscript{401} to Ala caused only a partial loss of SGK1 activation by these agents. These results are consistent with the conclusion that phosphorylation of Ser\textsuperscript{397} and to a lesser extent Ser\textsuperscript{401} is critical for SGK1 activity.

WNK1 and Akt1 Are Involved in Stimulation of SGK1 by H\textsubscript{2}O\textsubscript{2} and IGF1—We showed previously that Akt1 activity is required for SGK1 activation by WNK1 and that WNK1 activity is required for activation of SGK1 by IGF1 (17). To confirm the importance of these two proteins in SGK1 activation by H\textsubscript{2}O\textsubscript{2}, WNK1 and Akt1 were knocked down individually using RNA interference in cells expressing SGK1. As shown in Fig. 5A, decreased expression of endogenous WNK1 greatly reduced SGK1 activation by H\textsubscript{2}O\textsubscript{2}. Because Akt1 abundance is low in HeLa cells, Akt1 knockdown was conducted in both HeLa and MDA-MB-231 breast cells, which express more Akt1. IGF1 and H\textsubscript{2}O\textsubscript{2} were used to stimulate SGK1 in both cell types. As shown in Fig. 5B, the decrease in endogenous Akt1 led to a significant loss of SGK1 activation. These experiments support the earlier conclusion that both WNK1 and Akt1 are involved in SGK1 regulation by a variety of ligands. In HeLa cells, we noted an unexpected effect of knocking down Akt1 expression to decrease ERK1/2 activity. No similar loss of ERK1/2 activity was observed upon Akt1 knockdown in MDA-MB-231 cells.
The N Terminus of WNK1 Collaborates with Akt1 to Activate SGK1—As shown above and in Fig. 6A, no SGK1 activity was detected if it was expressed with wild-type Akt1. Moderate SGK1 activity was observed when it was coexpressed with WNK1-(1–491) or Myr-Akt1 in HeLa cells (Fig. 6A). However, when SGK1 was coexpressed with WNK1-(1–491) and Myr-Akt1 together, more activity was detected, which suggests that these two proteins work through different mechanisms to increase SGK1 activity. WNK1-(1–491) also increased the activity of full-length SGK1(S401D) (Fig. 6B).

SGK1 binds to the N-terminal 500 residues of WNK1 as determined by in vitro binding and co-immunoprecipitation experiments. Thr58 in the N terminus of WNK1 can be phosphorylated by Akt1 or SGK1 in vitro and is phosphorylated in cells (17, 18). We considered the possibility that WNK1 and Akt1 may also form a stable complex. Overexpressed WNK1-(1–491) co-immunoprecipitated with overexpressed Akt1 (Fig. 7, A and B). However, we were unable to find conditions to isolate a complex of endogenous proteins. The low abundance of endogenous Akt1 in HeLa cells may account for the lack of detectable interaction.

**DISCUSSION**

Here, we have shown that phosphorylation of a third site, Ser397, is essential for activation of SGK1 by coexpression with phosphorylation (15). Phosphorylation of Ser371 in p70 S6 kinase is essential for its activation. However, Thr450 in Akt was constitutively phosphorylated in serum-starved 293 cells; replacement with Ala did not block its activation by growth factors, suggesting that it is not required for Akt activation (11). Not only was Ser397 the predicted third conserved phosphorylation site in SGK1 (15), SGK1 purified from Sf9 cells for crystallographic studies was phosphorylated at both Ser397 and Ser401 (27), sites that were assumed in that study to be irrelevant to regulation of SGK1 activity. These sites were mutated prior to crystallization and led to an SGK1 structure with a disordered C terminus. This result is consistent with the possibility that phosphorylation of these two sites enhances the folding and stability of the SGK1 C terminus. Fig. 8 shows an overlay of the SGK1 structure (light blue) with a model of SGK1 based on the protein kinase A crystal structure (dark blue). In the SGK1 crystal structure, residues after Gly378 were disordered. The model suggests that residues after Gly378 wrap across the glycine-rich loop, placing Ser397 near the top of the structure. In the model, the C-terminal residues (green helix) fold to the back of the structure, placing the hydrophobic motif in its appropriate position near a pocket formed by α-helix C (largely disordered in the SGK1 structure) and β-strand 5.
Why might phosphorylation of the third conserved site be required for full activity of p70 S6 kinase and SGK1 but not Akt? The third conserved site was proposed to cooperate with the hydrophobic motif phosphate by binding to a phospho-Ser/Thr-binding site above the glycine-rich loop within the kinase domain. This would promote zipper-like association of the tail with the kinase domain. This interaction should juxtapose the hydrophobic motif with its binding site in the active form of the enzyme (15). A binding pocket in PDK1, which phosphorylates the activation loop in all three, has been reported to be essential for activation of p70 S6 kinase and SGK1 but not for Akt (28). Based on several types of data, phosphorylation of p70 S6 kinase and SGK1 at sites in their hydrophobic motifs is thought to create a high affinity docking site enabling PDK1 to bind and therefore phosphorylate the activation loop. In contrast, Akt, which has a pleckstrin homology domain, does not rely on this binding pocket for activation. Despite the high sequence similarity between SGK1 and Akt, SGK1 lacks a pleckstrin homology domain. Thus, how it is recruited to its site of activation must be distinct but is still unclear. Perhaps SGK1 depends more heavily than Akt on structural interactions with PDK1 to achieve full activation. Reasoning such as this may explain why additional sites of phosphorylation are important for activation of SGK1 and some other AGC kinases.

Although the third conserved sites were predicted in up to 26 AGC family proteins, the known phosphorylation mechanisms vary. In p70 S6 kinase, Ser371 is thought to be phosphorylated by mTOR, but in protein kinase C enzymes, most data suggest that autophosphorylation is the mechanism (24, 29, 30). We found that active Akt1 induces the phosphorylation of Ser397. Knockdown of Akt1 blocks SGK1 activation by IGF1 and H2O2. We
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considered the possibility that Akt may directly phosphorylate the third site in SGK1. Myr-Akt1 and wild-type ΔSGK1 or mutants were overexpressed in HeLa cells, and the immuno-precipitated proteins were mixed in kinase assays. No phosphorylation of SGK1 was detected (data not shown). The possibility that SGK1 autophosphorylates at Ser397 was also tested. ΔSGK1(5422D) was coexpressed with tagged ΔSGK1, but no activity change was observed. Thus, it seems most likely that Akt1 activates SGK1 through some indirect mechanism, such as recruiting mTOR, as has been suggested (7, 30), or other kinases that phosphorylate SGK1.

Ser397 is constitutively phosphorylated in SGK1 and also contributes to SGK1 activation. This residue is preceded by a hydrophobic residue and followed by a proline residue in the +1 position. These characteristics are also found in four phosphorylation sites in the autoinhibitory domain of p70 S6 kinase, which function to modulate kinase activity but are not required for full activity (31, 32). Understanding the function of Ser397 may shed light on regulation of Ser397 phosphorylation.

In many cell types, endogenous SGK1 is expressed at a very low level. Several groups have reported that ectopically introduced full-length SGK1 is poorly expressed but that truncated SGK1 (ΔSGK1) is expressed at much higher levels (22, 33). Most evidence indicates that the poor expression of full-length SGK1 is due to polyubiquitination and subsequent degradation by the 26 S proteasome; the target sequence lies in the N-terminal 60 residues of SGK1. Removal of this region nearly eliminates ubiquitin modification, significantly stabilizes the enzyme, and is thereby expected to increase SGK1 kinase activity and physiological functions. Nevertheless, we noted unexpected differences between ΔSGK1 and full-length SGK1 in side-by-side comparisons. Full-length SGK1 was poorly activated by Akt1 or IGF1 but strongly activated by mutation of Ser401 to Asp. These differences suggest that the N terminus of SGK1 may also have a regulatory role. The N-terminal region of SGK1 has been shown to bind to phosphoinositides (34). It seems possible that lipid binding may not only direct SGK1 to sites of action but may also serve to relieve an as yet unidentified autoinhibitory constraint.

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