THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 6, pp. 3453–3460, February 6, 2009

Regulation of a Third Conserved Phosphorylation Site in SGK1*

Received for publication, September 29, 2008, and in revised form, December 8, 2008 Published, JBC Papers in Press, December 9, 2008, DOI 10.1074/jbc.M807502200

Wei Chen, Yue Chen[1], Bing-e Xu[2], Yu-Chi Juang, Steve Stippec, Yingming Zhao[1], and Melanie H. Cobb[3]

From the Departments of Pharmacology and Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9041

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 Thr^{256} in the activation loop and Ser^{422} in the hydrophobic motif near the C terminus. Here, we report the identification of two additional phosphorylation sites, Ser^{397} and Ser^{401}. 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 Ser^{397}. Coexpression with active Akt1 increased the phosphorylation of Ser^{397} 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)[4] 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 Ser^{422} in a C-terminal region that extends from the core kinase domain, termed the hydrophobic motif. Ser^{422} can be phosphorylated by mTOR and perhaps DNA-dependent protein kinase and other protein kinases (7, 8). Phosphorylation of Ser^{422} transforms SGK1 into a substrate for the phosphoinositide-dependent protein kinase PDK1. PDK1 binds to the hydrophobic motif on SGK1, promoting phosphorylation of Thr^{256} 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 Thr^{58} (18). Mutation of Thr^{58} 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 Thr^{58} 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.

* This work was supported, in whole or in part, by National Institutes of Health Grant GM53032 (to M. H. C.) and "Innovative Technologies for the Molecular Analysis of Cancer" Program Grant CA107943 from NCI (to Y. Z.). This work was also supported by Welch Foundation Grant I1243 (to M. H. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Present address: Ben May Department for Cancer Research, University of Chicago, Chicago, IL 60637.

[2] Present address: Physician's Education Resource, Dallas, TX 75219.

[3] To whom correspondence should be addressed: Dept. of Pharmacology, University of Texas Southwestern Medical Center, 6001 Forest Park Rd., Dallas, TX 75390-9041. Tel.: 214-645-6125; Fax: 214-645-6124; E-mail: Melanie.Cobb@UTSouthwestern.edu.

[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.

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-Thr^{256} and phospho-Ser^{422} 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 Sigma. The anti-Akt1 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% CO_2. 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, cagacagugcaguauucacTT (sense) and gugaauacugcacugucugTT (antisense); and human Akt1, gaccgccucugcuuugucaTT (sense) and ugacaaagcagaggcggucTT (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 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride); detergent was omitted for co-immunoprecipitation experiments. Insoluble material was sedimented in a microcentrifuge for 15 min at 4 °C. Protein concentration was measured by the Bradford assay using bovine serum albumin as a standard. Soluble protein from each sample was resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk for 1 h at room temperature and then incubated with the appropriate antibodies.

Immunoprecipitation and In Vitro Protein Kinase Assays—Lysate protein (0.5 mg) was precleared with 50 μl of a 50% slurry of protein A-Sepharose beads for 1 h at 4 °C. The supernatant was incubated with the indicated antibody and 30 μl of a 50% slurry of protein A-Sepharose beads at 4 °C overnight. After three washes with 0.25 M Tris (pH 7.4), 1 M NaCl, 0.1% Triton X-100, and 0.1% sodium deoxycholate and one wash with 10 mM HEPES (pH 7.6), beads were incubated with the indicated substrates in 50 μl of kinase buffer (20 mM HEPES (pH 7.6), 5 μM ATP (5 μCi of [γ-^{32}P]ATP), 10 mM $MgCl_2$, and 10 mM β-glycerol phosphate) at 30 °C for 45 min.

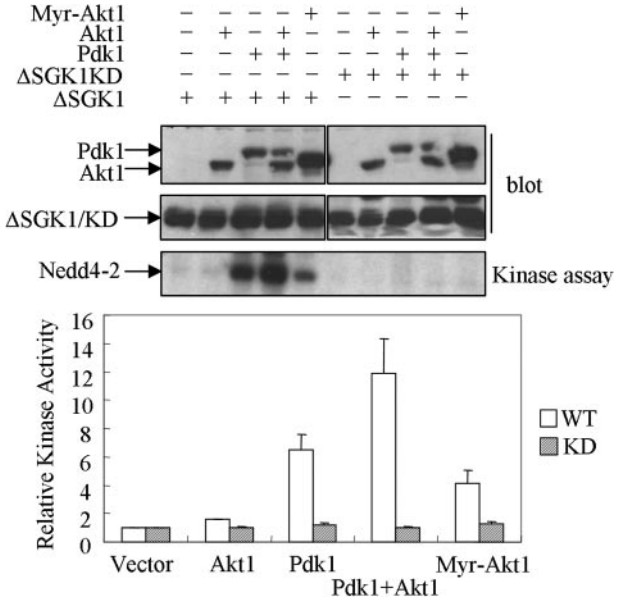

FIGURE 1. **SGK1 is activated by active Akt1.** Wild-type (*WT*) or kinase-dead (*KD*) ΔSGK1 was coexpressed with Myc-PDK1, HA-Akt1, or HA-Myr-Akt1 in HeLa cells. Protein expression was probed with anti-Myc, anti-FLAG, and anti-HA antibodies (*upper two panels*). Immunoprecipitated ΔSGK1 was assayed with GST-Nedd4-2 as substrate; shown are an autoradiogram and quantitation of three experiments (*lower panel*, plotted comparing wild-type SGK1 and kinase-dead ΔSGK1).

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).

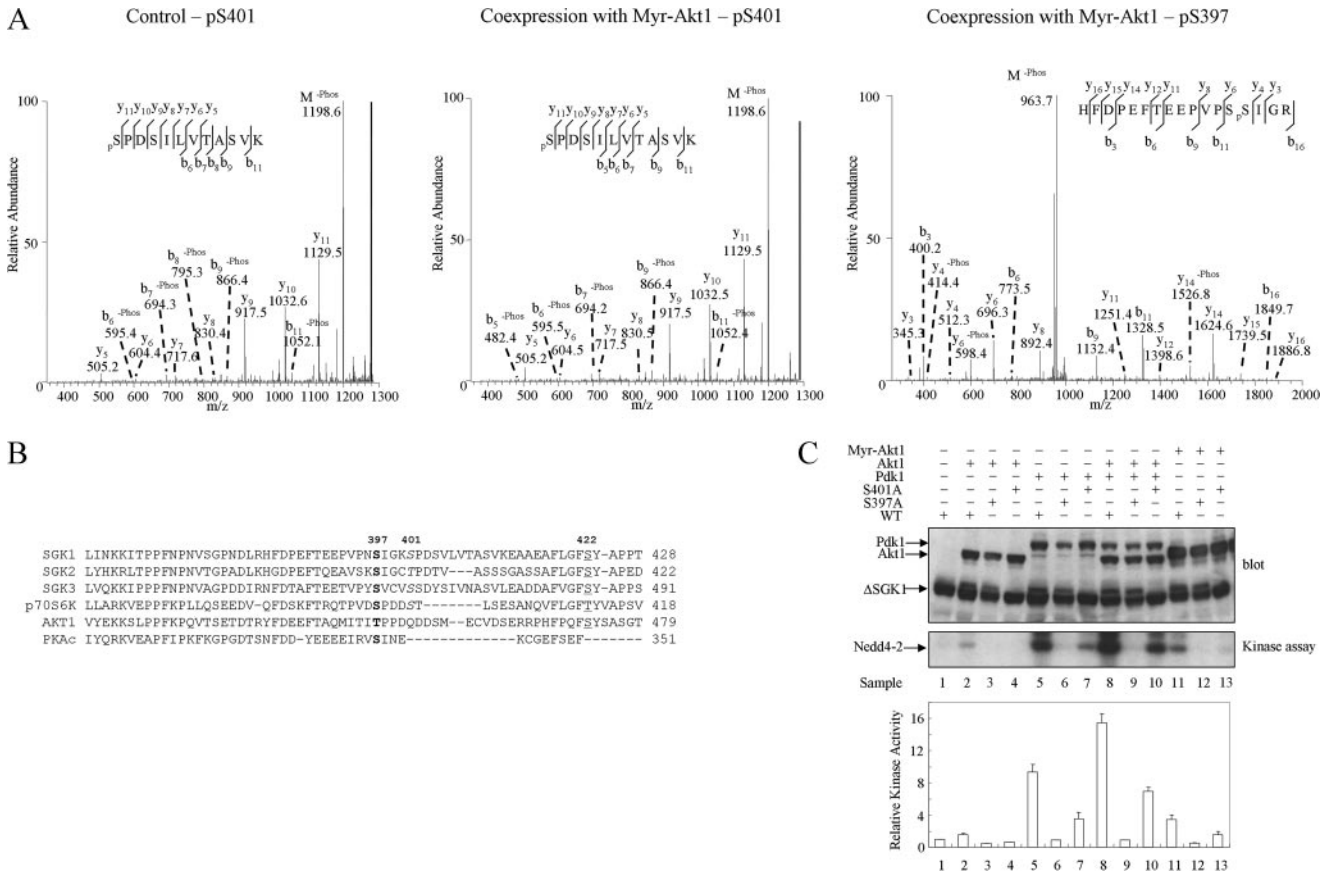

FIGURE 2. **Active Akt enhances phosphorylation of SGK1 Ser^{397}.** *A*, FLAG-SGK1 was coexpressed with vector or HA-Myr-Akt1 in HeLa cells. Immunoprecipitated SGK1 was subjected to mass spectrometry. Mass spectroscopic data of the phosphopeptide phospho-Ser^{401} in the control sample and phospho-Ser^{401} and phospho-Ser^{397} in samples from cells also expressing Myr-Akt1 are shown. *B*, shown is an amino acid sequence alignment of the tail region of selected AGC kinases. Conserved phosphorylation sites aligning with Ser^{397} are in *boldface*, and phosphorylation sites aligning with Ser^{401} are in *italics*. *PKAc*, protein kinase A. *C*, FLAG-ΔSGK1 or mutant ΔSGK1(S397A) or ΔSGK1(S401A) was coexpressed with Myc-PDK1, HA-Akt1, or HA-Myr-Akt1 in HeLa cells. Protein expression was probed with anti-Myc, anti-FLAG, and anti-HA antibodies. Immunoprecipitated ΔSGK1 proteins were assayed with GST-Nedd4-2 as substrate ($n = 4$). *WT*, wild-type.

Therefore, ΔSGK1 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-ΔSGK1 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. 2*A*). 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. 2*B*), 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 (S397A and S401A). As shown in Fig. 2*C*, SGK1(S397A) 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(S401A) 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. 3*A*). As expected, there was some reactivity of the S422D mutant with the anti-SGK1 phospho-Thr^{256} antibody, consistent with its increased kinase activity. Interestingly, the S422D mutant was also recognized by the anti-SGK1 phospho-Ser^{422} antibody. Mutation of Ser^{401} to Ala did not interfere with these enhancing effects of S422D. On the other hand, mutation of Ser^{397} to Ala prevented the SGK1 activity increase associated with the S422D mutant; the reduction could be detected not only by kinase assay but also by reduced phospho-

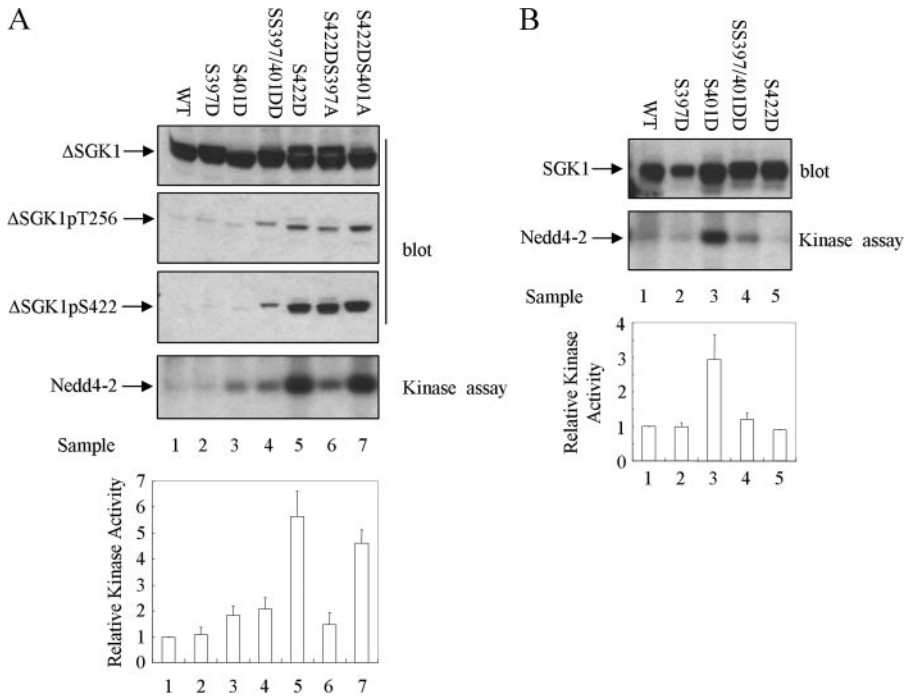

FIGURE 3. **Effects of mutating phosphorylatable residues to Ala or Asp.** FLAG-tagged ΔSGK1 (*A*) and SGK1 (*B*) and the indicated mutants of each were expressed in HeLa cells. Protein expression was probed with anti-FLAG, anti-SGK1 phospho-Thr^{256}, or anti-SGK1 phospho-Ser^{422} antibodies. Immunoprecipitated SGK1 proteins were assayed with GST-Nedd4-2 as substrate ($n = 3$ in *A* and *B*). *WT*, wild-type.

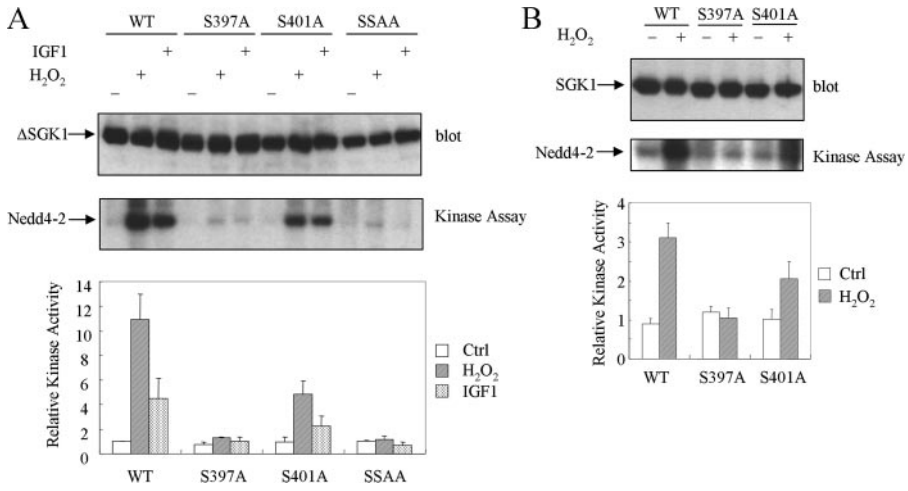

FIGURE 4. **Mutation of S397A blocks SGK1 activation by H_2O_2 and IGF1.** *A*, FLAG-ΔSGK1 or the indicated mutants were expressed in HeLa cells. Cells were untreated (−) or treated (+) with 2 mM H_2O_2 for 25 min or with 50 ng/ml IGF1 for 30 min before harvest. Protein expression was probed with the anti-FLAG antibody. Immunoprecipitated ΔSGK1 or mutants were assayed with GST-Nedd4-2 as substrate ($n = 4$). *B*, full-length FLAG-SGK1 or the indicated mutants were expressed in HeLa cells. Cells were untreated (−) or treated (+) with 2 mM H_2O_2 for 40 min. Protein expression was probed with the anti-FLAG antibody. Immunoprecipitated SGK1 or mutants were assayed with GST-Nedd4-2 as substrate ($n = 3$). *WT*, wild-type; *Ctrl*, control.

Thr^{256} reactivity. To determine whether acidic substitutions for Ser^{397} or Ser^{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^{397} Is Necessary for Stimulation of SGK1 by IGF1 and H_2O_2—SGK1 is activated by growth factors and cell stresses, including IGF1 and H_2O_2 (25, 26). H_2O_2 activated full-length SGK1 in HeLa cells much more than did IGF1 (Fig. 4*B* and data not shown). To detect if the phosphorylation of Ser^{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_2O_2. Mutation of Ser^{397} to Ala blocked stimulation of ΔSGK1 and SGK1 by either IGF1 or H_2O_2 (Fig. 4, *A* and *B*). Results were similar with the double S397A/S401A mutant. In contrast, mutation of Ser^{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^{397} and to a lesser extent Ser^{401} is critical for SGK1 activity.

WNK1 and Akt1 Are Involved in Stimulation of SGK1 by H_2O_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_2O_2, WNK1 and Akt1 were knocked down individually using RNA interference in cells expressing ΔSGK1. As shown in Fig. 5*A*, decreased expression of endogenous WNK1 greatly reduced SGK1 activation by H_2O_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_2O_2 were used to stimulate SGK1 in both cell types. As shown in Fig. 5*B*, 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.

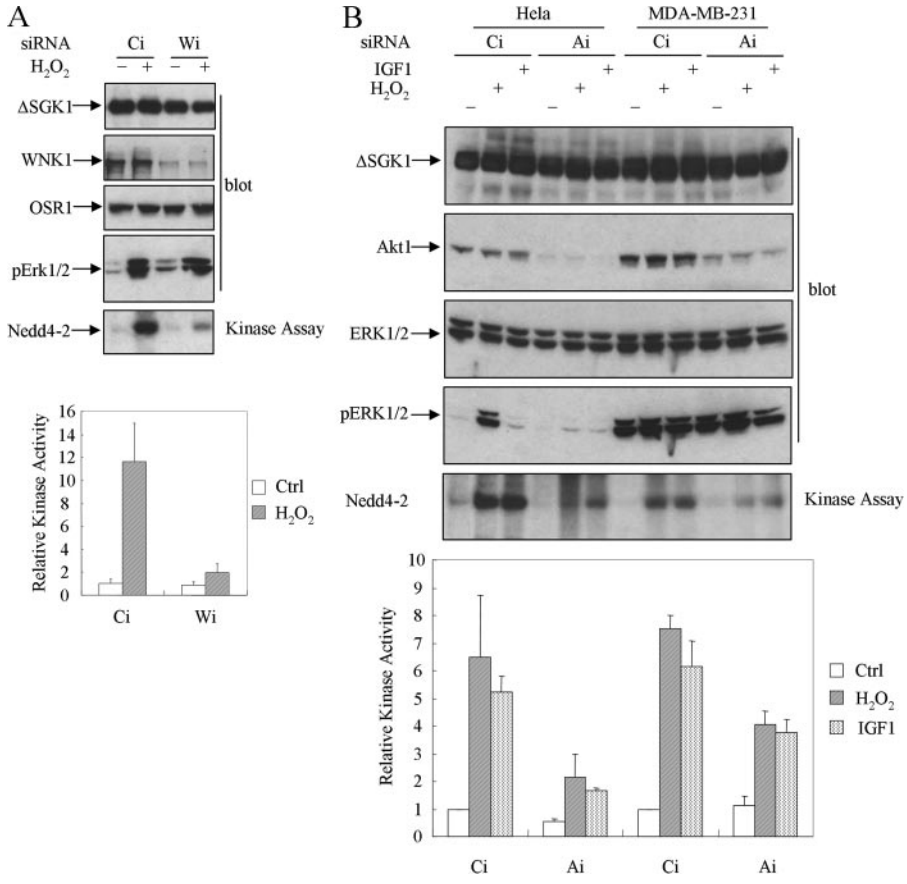

FIGURE 5. **WNK1 and Akt1 are involved in SGK1 activation by H_2O_2 and IGF1.** HeLa cells were cotransfected with plasmid encoding FLAG-ΔSGK1 and WNK1 (*Wi*), Akt1 (*Ai*), or scrambled (*Ci*) small interfering RNA (*siRNA*) oligonucleotides. Cells were untreated (−) or treated (+) with 2 mM H_2O_2 for 25 min or with 50 ng/ml IGF1 for 30 min before harvest. Lysates were probed with anti-FLAG, anti-WNK1, anti-Akt1, anti-phospho-ERK1/2, and either anti-OSR1 (*A*) or anti-ERK1/2 (*B*) antibodies. Immunoprecipitated ΔSGK1 was assayed with GST-Nedd4-2 as substrate. *A*, WNK1 knockdown; *B*, Akt1 knockdown ($n = 3$ in *A* and *B*). *Ctrl*, control.

The N Terminus of WNK1 Collaborates with Akt1 to Activate SGK1—As shown above and in Fig. 6*A*, 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. 6*A*). 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. 6*B*).

SGK1 binds to the N-terminal 500 residues of WNK1 as determined by *in vitro* binding and co-immunoprecipitation experiments. Thr^{58} 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, Ser^{397}, is essential for activation of SGK1 by coexpression with known regulators and for activation by IGF1 and H_2O_2. AGC protein kinases share relatively high sequence similarity within their kinase domains and may have related activation mechanisms. Studies revealed that phosphorylation of two highly conserved Ser/Thr residues located in the activation loop and the hydrophobic motif near the C terminus is indispensable for full activation of Akt, SGK1, and several other AGC family members, leading to the current paradigm for regulation of this family. Over a number of years, multiple other phosphorylation sites have also been identified in AGC kinases, but their roles in kinase regulation have not been so easily categorized. For example, Ser^{371} and Thr^{389} were identified in the linker region, and Ser^{411}, Ser^{418}, Thr^{421}, and Ser^{424} were found phosphorylated in the autoinhibitory domain of active forms of p70 S6 kinase (12, 13). Thr^{450} and Ser^{124} were found phosphorylated in Akt (11). From a recent comparison of sequences and structural data from several AGC family members, it was proposed that p70 S6 kinase (Ser^{371}) and Akt (Thr^{450}) each contain a third conserved regulatory site of phosphorylation (15). Phosphorylation of Ser^{371} in p70 S6 kinase is essential for its activation. However, Thr^{450} 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 Ser^{397} the predicted third conserved phosphorylation site in SGK1 (15), SGK1 purified from Sf9 cells for crystallographic studies was phosphorylated at both Ser^{397} and Ser^{401} (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 Gly^{378} were disordered. The model suggests that residues after Gly^{378} wrap across the glycine-rich loop, placing Ser^{397} 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.

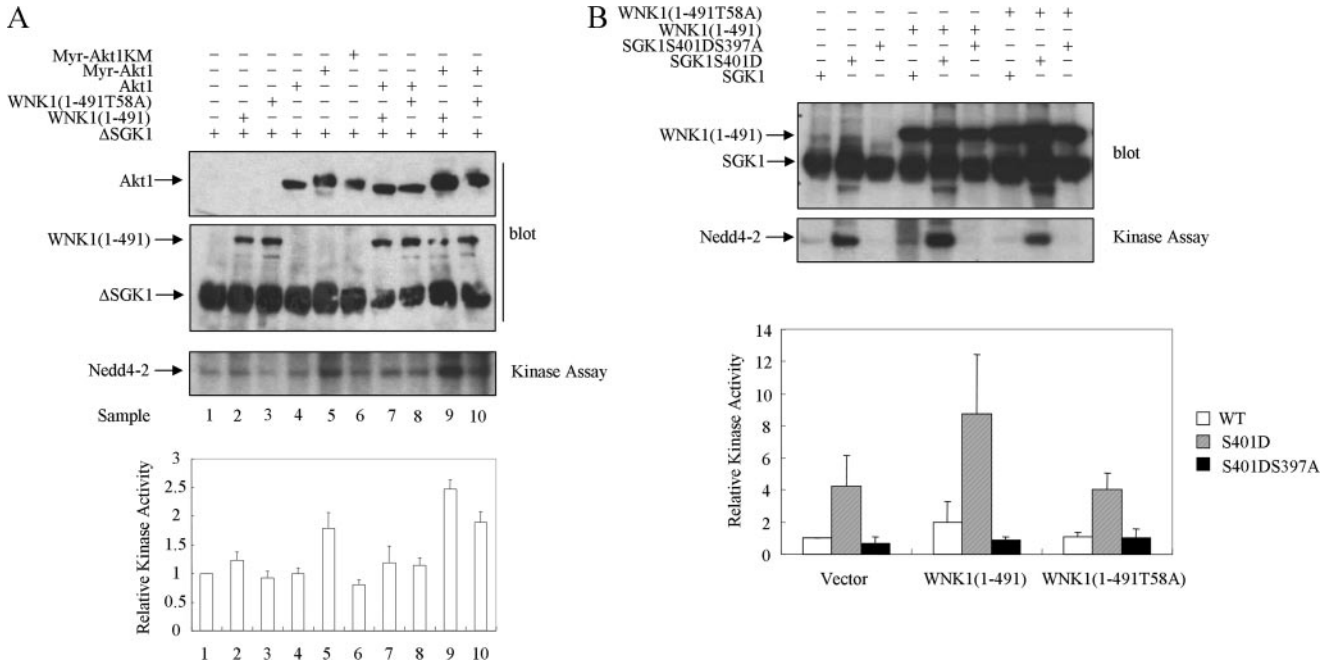

FIGURE 6. **WNK1 and Akt1 cooperate to activate SGK1.** *A*, FLAG-ΔSGK1 was coexpressed with Myc-WNK1-(1–491), Myc-WNK1-(1–491)(T58A), HA-Akt1, HA-Myr-Akt1, or HA-Myr-Akt1KM (kinase-dead) in different combinations in HeLa cells. Protein expression was probed with anti-Myc, anti-FLAG, and anti-HA antibodies. Immunoprecipitated ΔSGK1 was assayed with GST-Nedd4-2 as substrate ($n = 3$). *B*, FLAG-SGK1 (full-length) or the indicated mutants were coexpressed with Myc-WNK1-(1–491) or Myc-WNK1-(1–491)(T58A) in HeLa cells. Protein expression was probed with anti-Myc and anti-FLAG antibodies. Immunoprecipitated SGK1 proteins were assayed with GST-Nedd4-2 as substrate ($n = 4$). *WT*, wild-type.

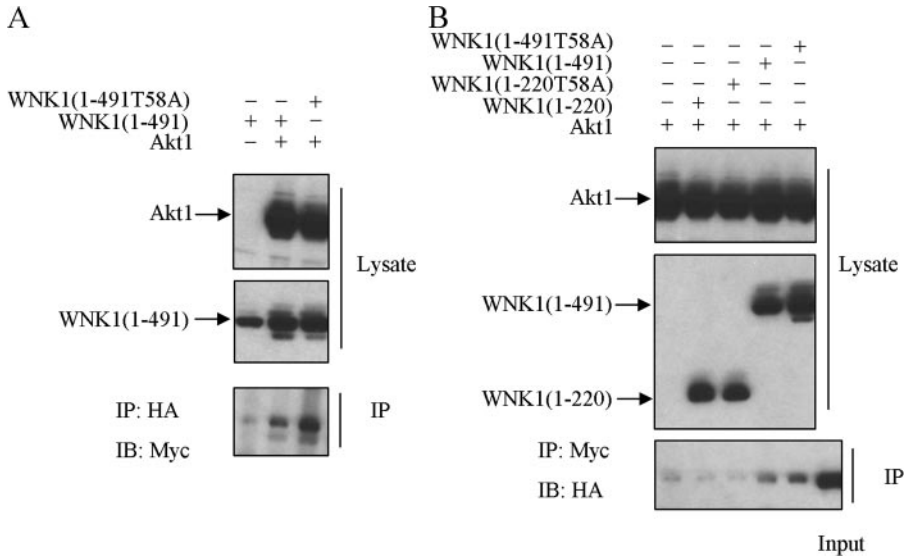

FIGURE 7. **Overexpressed WNK1-(1–491) co-immunoprecipitates with overexpressed Akt1.** HA-Akt1, Myc-WNK1-(1–491), Myc-WNK1-(1–491)(T58A), Myc-WNK1-(1–220), or Myc-WNK1-(1–220)(T58A) was coexpressed in HeLa cells in the indicated combinations. Cells were harvested in detergent-free lysis buffer and lysed by aspiration through a 22-gauge needle 30 times. Anti-HA (*A*) or anti-Myc (*B*) immunoprecipitates (*IP*) were washed with lysis buffer, resolved on gels, and immunoblotted (*IB*) with the anti-Myc or anti-HA antibody.

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, Ser^{371} 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 Ser^{397}. Knockdown of Akt1 blocks SGK1 activation by IGF1 and H_2O_2. We

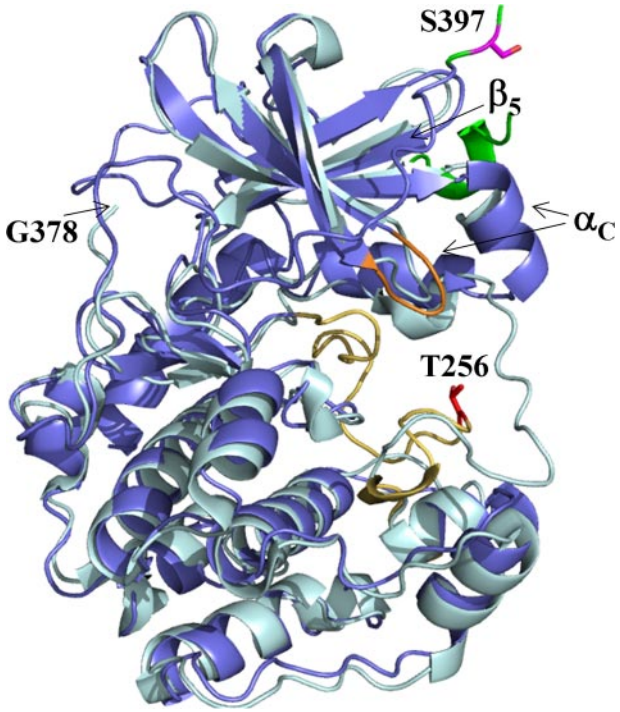

FIGURE 8. The figure shows the superposition of the SGK1 model (*dark blue*) with the SGK1 crystal structure (*light blue*; Protein Data Bank code 2r5t). The SGK1 model is based on the protein kinase A structure (Protein Data Bank code 1atp) using the homology modeling tool SWISS-MODEL (35). The activation loop, ATP-binding loop, and hydrophobic motif in the SGK1 model are colored *yellow*, *orange*, and *green*, respectively. Ser^{397} and Thr^{256} are shown as *sticks*. *Arrows* indicate the positions of Gly^{378}, α-helix C (α_C), and β-strand 5 (β_5).

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 immunoprecipitated proteins were mixed in kinase assays. No phosphorylation of SGK1 was detected (data not shown). The possibility that SGK1 autophosphorylates at Ser^{397} was also tested. ΔSGK1(S422D) 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.

Ser^{401} 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 Ser^{401} may shed light on regulation of Ser^{397} 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 Ser^{401} 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.

Acknowledgments—We thank Alexandra Newton (University of California, San Diego) for helpful discussions; Charles Heise, Staci Deaton, and Arif Jivan for suggestions and comments about the manuscript; Svetlana Earnest for technical assistance; and Dionne Ware for administrative assistance.

REFERENCES

1. Webster, M. K., Goya, L., Ge, Y., Maiyar, A. C., and Firestone, G. L. (1993) *Mol. Cell. Biol.* **13,** 2031–2040
2. Tessier, M., and Woodgett, J. R. (2006) *J. Cell. Biochem.* **98,** 1391–1407
3. Kamynina, E., and Staub, O. (2002) *Am. J. Physiol.* **283,** F377–F387
4. Wulff, P., Vallon, V., Huang, D. Y., Volkl, H., Yu, F., Richter, K., Jansen, M., Schlunz, M., Klingel, K., Loffing, J., Kauselmann, G., Bosl, M. R., Lang, F., and Kuhl, D. (2002) *J. Clin. Investig.* **110,** 1263–1268
5. Vallon, V., Huang, D. Y., Grahammer, F., Wyatt, A. W., Osswald, H., Wulff, P., Kuhl, D., and Lang, F. (2005) *Am. J. Physiol.* **289,** R395–R401
6. Lang, F., Bohmer, C., Palmada, M., Seebohm, G., Strutz-Seebohm, N., and Vallon, V. (2006) *Physiol. Rev.* **86,** 1151–1178
7. Hong, F., Larrea, M. D., Doughty, C., Kwiatkowski, D. J., Squillace, R., and Slingerland, J. M. (2008) *Mol. Cell* **30,** 701–711
8. Feng, J., Park, J., Cron, P., Hess, D., and Hemmings, B. A. (2004) *J. Biol. Chem.* **279,** 41189–41196
9. Gao, T., and Newton, A. C. (2002) *J. Biol. Chem.* **277,** 31585–31592
10. Gao, T., Toker, A., and Newton, A. C. (2001) *J. Biol. Chem.* **276,** 19588–19596
11. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998) *Oncogene* **17,** 313–325
12. Moser, B. A., Dennis, P. B., Pullen, N., Pearson, R. B., Williamson, N. A., Wettenhall, R. E., Kozma, S. C., and Thomas, G. (1997) *Mol. Cell. Biol.* **17,** 5648–5655
13. Saitoh, M., Pullen, N., Brennan, P., Cantrell, D., Dennis, P. B., and Thomas, G. (2002) *J. Biol. Chem.* **277,** 20104–20112
14. McCoy, C. E., Campbell, D. G., Deak, M., Bloomberg, G. B., and Arthur, J. S. (2005) *Biochem. J.* **387,** 507–517
15. Hauge, C., Antal, T. L., Hirschberg, D., Doehn, U., Thorup, K., Idrissova, L., Hansen, K., Jensen, O. N., Jorgensen, T. J., Biondi, R. M., and Frodin, M. (2007) *EMBO J.* **26,** 2251–2261
16. Xu, B., Stippec, S., Chu, P.-Y., Li, X.-J., Lazrak, A., Ortega, B., Lee, B. H., English, J. M., Huang, C.-L., and Cobb, M. H. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 10315–10320
17. Xu, B., Stippec, S., Lazrak, A., Huang, C.-L., and Cobb, M. H. (2005) *J. Biol. Chem.* **280,** 34218–34223
18. Vitari, A. C., Deak, M., Collins, B. J., Morrice, N., Prescott, A. R., Phelan, A., Humphreys, S., and Alessi, D. R. (2004) *Biochem. J.* **378,** 257–268
19. Xu, B., English, J. M., Wilsbacher, J. L., Stippec, S., Goldsmith, E. J., and Cobb, M. H. (2000) *J. Biol. Chem.* **275,** 16795–16801
20. Chen, W., Yazicioglu, M., and Cobb, M. H. (2004) *J. Biol. Chem.* **279,** 11129–11136
21. Shu, H., Chen, S., Bi, Q., Mumby, M., and Brekken, D. L. (2004) *Mol. Cell. Proteomics* **3,** 279–286
22. Brickley, D. R., Mikosz, C. A., Hagan, C. R., and Conzen, S. D. (2002) *J. Biol. Chem.* **277,** 43064–43070

23. Belova, L., Sharma, S., Brickley, D. R., Nicolarsen, J. R., Patterson, C., and Conzen, S. D. (2006) *Biochem. J.* **400,** 235–244
24. Newton, A. C. (2003) *Biochem. J.* **370,** 361–371
25. Kobayashi, T., and Cohen, P. (1999) *Biochem. J.* **339,** 319–328
26. Leong, M. L., Maiyar, A. C., Kim, B., O'Keeffe, B. A., and Firestone, G. L. (2003) *J. Biol. Chem.* **278,** 5871–5882
27. Zhao, B., Lehr, R., Smallwood, A. M., Ho, T. F., Maley, K., Randall, T., Head, M. S., Koretke, K. K., and Schnackenberg, C. G. (2007) *Protein Sci.* **16,** 2761–2769
28. Biondi, R. M., Kieloch, A., Currie, R. A., Deak, M., and Alessi, D. R. (2001) *EMBO J.* **20,** 4380–4390
29. Parekh, D. B., Ziegler, W., and Parker, P. J. (2000) *EMBO J.* **19,** 496–503
30. Parekh, D., Ziegler, W., Yonezawa, K., Hara, K., and Parker, P. J. (1999) *J. Biol. Chem.* **274,** 34758–34764
31. Mukhopadhyay, N. K., Price, D. J., Kyriakis, J. M., Pelech, S., Sanghera, J. S., and Avruch, J. (1992) *J. Biol. Chem.* **267,** 3325–3335
32. Han, J. W., Pearson, R. B., Dennis, P. B., and Thomas, G. (1995) *J. Biol. Chem.* **270,** 21396–21403
33. Bogusz, A. M., Brickley, D. R., Pew, T., and Conzen, S. D. (2006) *FEBS J.* **273,** 2913–2928
34. Pao, A. C., McCormick, J. A., Li, H., Siu, J., Govaerts, C., Bhalla, V., Soundararajan, R., and Pearce, D. (2007) *Am. J. Physiol.* **292,** F1741–F1750
35. Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. (2006) *Bioinformatics* **22,** 195–201