Role of the Phox Homology Domain and Phosphorylation in Activation of Serum and Glucocorticoid-regulated Kinase-3*  

Maude Tessier and James R. Woodgett  
From the Samuel Lunenfeld Research Institute and Department of Medical Biophysics, University of Toronto, Toronto, Ontario MSG 1X5, Canada

Serum and glucocorticoid-regulated kinases (SGKs) form a family of serine/threonine protein kinases that exhibit structural and sequence similarity to the protein kinase B (PKB)/Akt family. The major difference between these two families is the absence of a lipid-binding, pleckstrin homology domain in the SGKs. Despite the absence of the pleckstrin homology domain, activation of the three human isoforms is, like PKB, dependent upon the phosphatidylinositol 3'-kinase (PI(3)K) pathway that is induced by growth factors and mitogens. Full-length SGK3 contains a complete Phox homology (PX) domain that targets the protein to endosomes. Both a functional PX domain and PI(3)K activation are necessary for phosphorylation of SGK3 at two regulatory sites (Thr-320 and Ser-486) and subsequent induction of kinase activity. PDK1 phosphorylates endosome-associated SGK3 at Thr-320, whereas diversion of SGK3 to the plasma membrane, where PDK1 normally activates PKB, interferes with PDK1 phosphorylation of SGK3. A chimeric protein in which the carboxyl-terminal hydrophobic motif (HM) of SGK3 has been exchanged for the HM of PRK2 is constitutively active. Finally, we demonstrate that SGK3 activation becomes PX domain-independent once the HM is phosphorylated. Taken together, these data indicate that the targeting of SGK3 to endosomes, mediated by its PX domain, is essential for proper SGK3 activation, likely due to co-localization of SGK3 with an endosomal, PI(3)K-dependent and staurosporine-sensitive HM kinase.
Regulation of SGK3 by Its PX Domain

SGK3 (fwd, gtctagaatgcagagatccacagtggc; rev, ggtacctcagaaaagaaagttcctcggaggg). The PCR product was then subcloned into a p3XFLAG-CMV-14 mammalian expression vector (Sigma) in which a myristoylation sequence had been introduced in the HindIII and KpnI sites. The portion of the wild type SGK3 cDNA corresponding to the PX domain (amino acids 1–136) was amplified by PCR. BamHI and NotI sites were introduced (fwd, ggtacctgcaaggatacaccatgg; rev, gcggccgccttttctcctcatttc), and the amplified product was subsequently subcloned into pGEX-4T-1 (Amersham Biosciences). SGK3-PRK2 was cloned using an adaptation of the Quick-Change (Stratagene) site-directed mutagenesis (8). A standard PCR was carried out with a PRK2 EST (Image clone ID 2124101; GenBank™ accession number AI633689; I.M.A.G.E. consortium, ATCC) as template. The HM of PRK2 (EEQEMFRDFDY-IADW) was amplified by using long primers that contained homologous sequences to SGK3 at their 5’ and 3’ ends.

**Experimental Procedures**

**Cloning of Full-length Human SGK3 and Generation of Mutants**—The human EST data base at NCBI was interrogated using CISK cDNA as a query. A human EST spanning the amino terminus and the kinase domain of CISK (Clone ID 4391699) was obtained from I.M.A.G.E. Consortium in pCMV-Sport6. Sequencing confirmed the presence of a full-length human SGK3 open reading frame in this EST. NotI and BamHI sites were introduced at the 5’ and 3’ ends of SGK3 using PCR (fwd, gcggccgcgtcacaagatcaccatgg; rev, ggtacctcagaaaagaaagttcctcggaggg) and subcloned into the p3XFLAG-CMV-10 mammalian expression vector (Sigma). Point mutations in SGK3 were generated by site-directed mutagenesis (QuickChange, Stratagene). Myristoylated SGK3 was generated by introducing XbaI and BamHI sites at the 5’ and 3’ ends of wild type sequences from the HM of PRK2 at their 3’ end (PRK2-1, gtatctttgctcatttctagtagtgaacaaagtagctttcgg; PRK2-2, ctggatgcaccacccggagctcttaccaaatcgaatg). This PCR product was separated on an agarose gel and eluted with PerfectPrep Gel Cleanup kit (Eppendorf). The PCR product (150 ng) then served as a master primer in a “QuickChange” PCR using 50 ng of SGK3 in p3XFLAG CMV10 (Sigma) as template. This yielded a “mutagenized” plasmid where nucleotides 1009–2424 were derived from SGK3 and 2425–2499 were from the HM of PRK2. The mutagenized plasmid was isolated by digesting the PCR for 3 h with DpnI and by transforming Top10 chemically competent cells (Invitrogen). All mutants were sequence-verified.

**In Vitro Translation**—Linked in vitro transcription and translation was carried out on EST (Clone ID 4391699) in pCMV-Sport6 from the sp6 bacteriophage promoter. PROTEInscript™ II from Ambion was used according to the manufacturer’s directions.

**Cell Culture and Transfection**—HEK293 cells and Rat1 fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C, 5% CO₂, and humidity. HEK293 cells were plated onto 35-mm diameter dishes and transfected with 500 ng of plasmid DNA using Lipofectamine 2000 (Invitrogen). The following day, the transfection medium was removed and replaced with complete Dulbecco’s modified Eagle’s medium. 16 h prior to treatments, cells were washed and serum-starved in Dulbecco’s modified Eagle’s medium without fetal bovine serum. After serum starvation, cells were untreated or treated with a PI3K agonist.
Regulation of SGK3 by Its PX Domain

(0.1 mM pervanadate, 15 min), with PI3K inhibitors (25 nM LY294002, 15 min), or with kinase inhibitors (1 μM staurosporine (Sigma), 15 min). Cells were lysed in Gentle Soft lysis buffer (100 mM NaCl, 20 mM Pipes, pH 7.0, 0.5% Nonidet P-40, 0.05% 2-mercaptoethanol, 5 mM EDTA, 50 mM NaF, 100 mM phosphoric acid, and the extent of 32P incorporation was determined by liquid scintillation counting. Results shown represent means ± S.E. Results shown are from three independent experiments.

Immunoprecipitation and Immunoblotting—10 μl of a 50% solution of Gamma Bind-Sepharose (Amersham Biosciences) and 0.4 μg of M2 FLAG antibody (Sigma) were added to lysates. Samples were incubated for 3 h at 4 °C. The immunoprecipitates were washed three times with cold Gentle Soft lysis buffer. Lysates/immunoprecipitates were boiled with SDS-containing sample buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 30 min in 4% skim milk and probed with the appropriate antibody overnight at 4 °C as follows: FLAG M2 antibody (Sigma), Myc antibody (Santa Cruz Biotechnology), phospho-Thr-320 SGK3 antibody (Cell Signaling Technologies), and phospho-PDK1 docking antibody (Cell Signaling Technologies). Membranes were incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit or antimouse antibodies at room temperature for 1 h. Proteins were visualized using ECL according to the manufacturers' protocol (Pierce).

Kinase Assays—HEK293 cells immunoprecipitates were washed five times with cold Gentle Soft lysis buffer and twice with cold Kinase buffer (10 mM MgCl2, 50 mM Tris-Cl, pH 7.5, 1 mM EGTA). Kinase reactions (0.1 mM ATP, [γ-32P]ATP, 5 μCi/sample, kinase buffer, 0.6 μM Cross-tide peptide substrate) were carried out in a 50-μl volume at 30 °C for 30 min. Half of the kinase reaction volume was spotted onto P81 chromatography paper (Whatman); the other half was analyzed by immunoblotting. P81 papers were repeatedly washed in 1% phosphoric acid, and the extent of 32P incorporation was determined by liquid scintillation counting. Results shown are combined data from three independent experiments.

Protein:Lipid Overlay Assay—The pGEX-4T-1 PX domain of SGK3 and R90A mutant were transformed into BL21 Gold bacteria (Stratagene). The recombinant GST–PX fusion proteins were induced with 0.4 mM isopropyl-1-thio-β-d-galactopyranoside and grown at 30 °C for 5 h. Cells were harvested by centrifugation at 10,000 × g for 10 min and placed at −80 °C overnight. Fusion proteins were purified using the BugBuster GST bind purification kit (Novagen) according to the manufacturer's instructions. Purity and yield of the fusion proteins were assessed by SDS-PAGE. PIPStrips (Echelon) were blocked for 1 h in TBST (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 3% fatty acid-free BSA (Sigma). The fusion proteins (2 μg/ml) in TBST with 3% fatty acid-free BSA were incubated with the blocked PIPstrip overnight at 4 °C. The strips were washed three times for 10 min with TBST and incubated for 1 h at room temperature with 6:2000 anti-GST (B-14) antibody (Santa Cruz Biotechnology) in TBST with 3% fatty acid-free BSA. The strips were washed three times for 10 min with TBST and incubated for 1 h at room temperature with 1:5000 anti-mouse IgG horseradish peroxidase (Roche Applied Science) in TBST with 3% fatty acid-free BSA. The strips were washed in TBST with 3% fatty acid-free BSA before ECL detection (Pierce).

Immunofluorescence—Rat1 fibroblasts were grown on glass coverslips and transfected using Lipofectamine 2000 (Invitrogen). For one experiment, Alexa 594-conjugated transferrin (Molecular Probes) was incubated on the cells at 37 °C for 30 min. Cells were fixed in 3% parafomaldehyde and permeabilized in 0.2% Triton X-100 in PBS. Cells were incubated with anti-FLAG M2 antibody (Sigma), anti-EEA1 (Calbiochem) and then with anti-mouse/rabbit anti-immunoglobulin antibody labeled with Oregon Green 488 and Texas Red (Molecular Probes), respectively. Cells were stained with 4,6-diamidino-2-
phenylindole, and the specimens were mounted in ProLong medium (Molecular Probes). Immunofluorescence was observed under a Zeiss LSM510 laser scanning confocal microscope, and images were captured with LSM5 software.

RESULTS

The PX Domain of Human SGK3 Binds PI(3)P and Targets SGK3 to Endosomes—We retrieved a human EST of mammary epithelial origin that spanned from the PX domain of CISK to its kinase domain (Clone ID 4391699) following a BLAST query. The EST contained the previously reported human SGK3 sequence as well as a complete PX domain, the sum of which we now consider to be full-length SGK3. The reported SGK3 sequence produced a 53-kDa protein, whereas the EST encoding full-length SGK3 is predicted to yield a 65-kDa protein. The in vitro transcribed protein was primarily composed of a 65-kDa product (Fig. 1A), indicating that translation is initiated from the upstream methionine and thus the protein includes a complete PX domain (Fig. 1A).

A GST fusion protein comprising the PX domain of SGK3 (amino acids 1–136) was incubated with a membrane spotted with phospholipids, and specific binding was detected using an anti-GST antibody. This protein lipid overlay (PLO) assay has been used extensively to characterize the lipid binding specificity of proteins (9). The PX domain of SGK3 bound strongly and preferentially to PI(3)P, and to a lesser degree to PI(4)P, PI(5)P,
Regulation of SGK3 by Its PX Domain

PI(3,4)P₂ and PI(3,5)P₂ (Fig. 1B). Mutation of Arg-90, a conserved residue within a positively charged pocket required for phosphatidylinositol head group binding (10), abolished binding to the phospholipids (Fig. 1B). These results demonstrate that the PX domain of human SGK3 is functional and that it preferentially interacts with PI(3)P. This monophosphate is thought to arise from the phosphorylation of phosphatidylinositol by type III PI3K. Transfection of Rat1 cells with wild type SGK3 followed by confocal immunofluorescence detection revealed an endosomal localization when compared with staining for EEA1 (endogenous early endosomal antigen-1), a marker of the endosomal compartment (Fig. 1C). The majority of the PI(3)P in mammalian cells was localized to endosomes (7, 11), and the localization data confirm the PLO assay.

**Human SGK3 Activity Is PI3K-dependent, whereas Its Localization Is PI3K-independent**—Wild type SGK3 was transiently transfected into HEK293 cells, which were then treated with pervanadate, an inhibitor of protein-tyrosine phosphatases and a potent agonist of PI3K, or pretreated with LY294002, an inhibitor of PI3K, immunoprecipitated, and subjected to an in vitro kinase assay (Fig. 2A). A low level of kinase activity was present upon transfection with wild type SGK3. Activity was stimulated ~20-fold upon treatment with pervanadate, and this induction was blocked by pretreatment of the cells with LY294002, confirming the PI3K dependence of SGK3 activation. The phosphorylation state of SGK3 was monitored using a phospho-Thr-320 antibody to indicate activation loop site phosphorylation and using a phospho-PDK1-docking motif antibody to probe immunoprecipitated SGK3 for the state of HM phosphorylation (Ser-486) (see supplemental Fig. 1). Immunoblotting revealed that pervanadate triggered phosphorylation at both sites and that pretreatment with LY294002 inhibited phosphorylation of both (Fig. 2, B and C). Hence, the in vivo phosphorylation state of SGK3 paralleled its kinase activity. We attempted to stimulate SGK3 phosphorylation with other PI3K agonists but failed (data not shown). This result suggests that PI3K activation is necessary but not sufficient for SGK3 phosphorylation. Immunofluorescence analysis of Rat1 cells expressing wild type SGK3 showed that even upon pervanadate-induced activation, SGK3 remains endosomal (Fig. 2D, top panel). Treatment of Rat1 SGK3 cells with increasing concentrations of LY294002 yielded enlarged endosomes, a previously reported phenomenon ascribed to the role of PI3K in endosome fusion. SGK3 was found to co-localize with transferrin, a PI3K-insensitive endosomal marker (21, 23), in the presence of LY294002, suggesting that SGK3 localization is PI3K-independent (Fig. 2D).

**Role of PX Domain in SGK3 Activity and Localization**—To determine whether, in addition to conferring PI3K dependence, the PX domain also contributed to SGK3 activation and localization, we performed immunofluorescence analysis on Rat1 cells expressing the R90A mutant of SGK3. This mutant harbors a mutation within the phosphoinositide binding pocket that abrogates its binding to phosphoinositides. This mutant demonstrated largely cytoplasmic and some nuclear localization, a previously reported phenomenon ascribed to the role of PI3K in endosome fusion, the PX domain also contributed to SGK3 activation and localization, we performed immunofluorescence analysis on Rat1 cells expressing wild type SGK3. This mutant showed that even upon pervanadate-induced activation, SGK3 remains endosomal (Fig. 2D, top panel). Treatment of Rat1 SGK3 cells with increasing concentrations of LY294002 yielded enlarged endosomes, a previously reported phenomenon ascribed to the role of PI3K in endosome fusion. SGK3 was found to co-localize

**FIGURE 3. Human SGK3 activity and localization are PX domain-dependent.** A, immunofluorescence was carried out as explained in Fig. 2 on Rat1 cells transfected with FLAG-SGK3 or R90A FLAG-SGK3. B, HEK293 cells were transfected with wild type (WT) SGK3 or an R90A mutant SGK3 and treated with or without 0.1 mM pervanadate. Lysates/immunoprecipitates (IP) were subjected to immunoblot analysis using the indicated antibodies. DAPI, 4,6-diamidino-2-phenylindole.

**TABLE 1**

|                 | SGK3       | R90A SGK3 |
|-----------------|------------|-----------|
| Pervanadate     | -          | +         |
| α Phospho T320  | -          | +         |
| IP: M2 FLAG     | -          | +         |
| α Phospho PDK1 docking | - | + |
| α M2 FLAG       | -          | +         |
Regulation of SGK3 by Its PX Domain

Myristoylation Is Insufficient for SGK3 Phosphorylation—
PDK1 phosphorylates PKB at the plasma membrane, and this event can be promoted by fusing a myristoylation (myr) signal to the amino-terminal domain of PKB (13). Indeed, this is the basis for retroviral activation of v-AKT whereby the fusion of the kinase to the myristoylated gag protein increases the amount of the kinase at the membrane where it encounters sufficient PDK1 to become activated (14). To determine whether the requirement for endosome localization for activation could be circumvented by targeting SGK3 to the plasma membrane, we generated a version of wild type SGK3 in which the myristoylation signal of c-Src was fused to the amino terminus (15). Despite the plasma membrane targeting signal, immunofluorescence showed that myr-SGK3 remained localized to endosomes (Fig. 4A) suggesting that the PX domain is dominant over the myristoylation signal. To counter this, the R90A mutation was introduced into the PX domain of myr-SGK3. In the context of the R90A mutation, the myr-SGK3 mutant was partially localized to the plasma membrane (Fig. 4A). However, despite this re-localization, the R90A myr-SGK3 was not phosphorylated at either the activation loop or the hydrophobic motif sites in response to stimulation (Fig. 4B). By contrast, myristoylated wild type SGK3 that was still endosome-bound was phosphorylated similarly to the nonmyristoylated SGK3. These data suggest that localization of SGK3 to endosomes is a prerequisite for its activation of SGK3 (see below).

Phosphorylation of SGK3 at Thr-320 by Ectopic PDK1—Based on the PI3K dependence of SGK3 activity and on knowledge gained from studies of PKB phosphorylation (13), we tested the role of PDK1 in activation of SGK3. HEK293 cells were co-transfected with wild type SGK3 and with wild type or various mutant PDK1 cDNAs, and phosphorylation of SGK3 was monitored by immunoblotting. Ectopic expression of PDK1 induced Thr-320 phosphorylation (Fig. 5A). Mutation of PDK1 within its “PIF pocket” (L155E) prevents docking of PDK1 with the hydrophobic motif in its AGC kinase substrates (16, 17). The L155E PDK1 mutant failed to phosphorylate SGK3 on Thr-320 (Fig. 5B), confirming that PDK1 relies on PIF pocket docking to phosphorylate SGK3. Inhibition of PI3K signaling with LY294002 or wortmannin failed to block phosphorylation of Thr-320 in the presence of ectopic PDK1, indicating that over-expression of this upstream kinase overcomes the requirement for PI3K (Fig. 5C).

A Staurosporine-sensitive Kinase Phosphorylates the Hydrophobic Motif of SGK3—To gain insight into the identity of the kinase(s) responsible for the hydrophobic motif phosphorylation (Ser-486), cells expressing wild type SGK3 and PDK1 were treated with staurosporine, a broad spectrum kinase inhibitor known to inhibit, among other protein kinases, PDK1 activity (18). As shown in Fig. 6A, staurosporine inhibited both Thr-320 and Ser-486 phosphorylation. This result suggests that staurosporine-sensitive kinase activities are responsible for both the activation loop and the hydrophobic motif phosphorylation. The importance of SGK3 kinase activity for its own localization and phosphorylation was evaluated using a kinase-inactive (K191M) SGK3 mutant. This mutant demonstrated normal endosomal compartment localization indicating this property of SGK3 is independent of its catalytic activity (Fig. 6B). Immunoblot analysis of HEK293 cells expressing wild type and K191M SGK3 showed that the kinase-dead mutant is not phosphorylated at either Thr-320 or Ser-486, even in the presence of pervanadate (Fig. 6C). These data suggest that the in vivo phosphorylation of SGK3 is mediated in part by PDK1 and in part by a staurosporine-sensitive kinase that could be PDK1, SGK3, or another unidentified kinase that is dependent upon some function of SGK3 (see “Discussion”).
Generation of a Constitutively Active Mutant of SGK3—To determine the importance of phosphorylation of the hydrophobic motif in SGK3 regulation, we generated a chimeric protein where the SGK3 HM was replaced with the HM of PRK2 (PKC-related kinase 2). This chimeric mutant was termed SGK3-PRK2 (Fig. 7A). PRK2 is an unusual AGC kinase in that it possesses an aspartic acid instead of a phosphorylatable residue in its HM. Presumably, the presence of the charged amino acid in the HM of PRK2 mimics the phosphorylated state of the HM of proteins such as PKB and SGK3. A peptide encompassing the HM of PRK2 was found to tightly interact with PDK1 and was termed PIF (19). In addition, a fusion of PKB/H9252 with the HM of PRK2 was exploited previously in crystallographic studies to obtain a stable conformation representing activated PKB (20, 21). We first tested the effect of the PRK2 HM fusion on SGK3 activity by monitoring its T-loop phosphorylation in extracts of HEK293 cells that transiently expressed the kinase fusion mutant (Fig. 7B). In the absence of the PI3K agonist, SGK3-PRK2 was significantly phosphorylated on Thr-320. The level of Thr-320 phosphorylation was further increased by treatment with pervanadate, likely reflecting additional recruitment of PDK1 under these conditions. LY294002 failed to block phosphorylation of Thr-320 of SGK3-PRK2. These results suggested that the SGK3-PRK2 mutant is constitutively active, and this was tested by measuring activity in an in vitro kinase assay using lysates of SGK3-PRK2-expressing cells. These experiments demonstrated that SGK3-PRK2 expressed activity in the absence of a PI3K agonist (Fig. 7C). Activity was increased slightly by pervanadate treatment but was largely unaffected by administration of a PI3K inhibitor. Of note, a mutant of SGK3 in which Ser-486 was mutated to aspartic acid was also made. That mutant did not yield a constitutively active kinase, primarily because of the unstable nature of the resultant protein. Replacing the aspartic acid corresponding to Ser-486 in SGK3-PRK2 with an alanine abrogated Thr-320 phosphorylation (data not shown), highlighting the importance of an acidic charge within the HM for activation of SGK3. SGK3-PRK2 exhibited an endosomal localization as determined by immunofluorescence (Fig. 7D). Finally, we tested the effect of SGK3-PRK2 expression on phosphorylation on one of the known targets of SGK3, GSK3 (glycogen synthase kinase 3). GSK3 phosphorylation was increased with expression of SGK3-PRK2, indicating that SGK3-PRK2 is an active kinase that despite the modification in the HM retains the specificity of the wild type protein (Fig. 7E). Taken together, these results demonstrate that replacing SGK3 HM with the HM of PRK2 yields a PI3K-independent kinase without affecting intracellular targeting or downstream functioning.

Phosphorylation of Thr-320 Is PX Domain-independent Once Ser-486 Is Phosphorylated—To investigate the relative roles of the PX domain and phosphorylation of the HM in SGK3 activation, we generated a mutant of SGK3-PRK2 in which the PX were treated with vehicle or with 0.1 mM pervanadate. Cell lysates were analyzed by immunoblot analysis as above. C, HEK293 cells were co-transfected with FLAG-SGK3 and wild type myc-PDK1. Cells were treated with 0.1 mM pervanadate or 25 μM LY294002 as indicated, and lysates were analyzed by immunoblot analysis as above.

---

**FIGURE 5.** Exogenous PDK1 renders Thr-320 phosphorylation-independent of PI3K. A, HEK293 cells were co-transfected with FLAG-SGK3 and either wild type myc-PDK1, myr-myc PDK1, or myc-KD PDK1. Some cells were untreated and some treated with 0.1 mM pervanadate. Cell lysates were analyzed by immunoblot analysis using the indicated antibodies. B, HEK293 cells were co-transfected with FLAG-SGK3 and either wild type myc-PDK1 or myc-L155E PDK1. Cells were treated with vehicle or with 0.1 mM pervanadate. Cell lysates were analyzed by immunoblot analysis as above. C, HEK293 cells were co-transfected with FLAG-SGK3 and wild type myc-PDK1. Cells were treated with vehicle or with 0.1 mM pervanadate or 25 μM LY294002 as indicated, and lysates were analyzed by immunoblot analysis as above.
SGK3 was disabled by the R90A mutation. This mutant and the respective control (R90A SGK3-PRK2D→A, aspartic acid residue in the HM mutated to alanine) are not localized to endosomes as shown by immunofluorescence confocal microscopy (Fig. 8A). We then monitored the phosphorylation of Thr-320 in the context of a “phosphorylated” HM and a non-functional PX domain. Immunoblotting revealed that R90A SGK3-PRK2 was still phosphorylated at Thr-320, despite the disabled PX domain (Fig. 8B). These data suggest that the activation loop phosphorylation of SGK3 becomes independent of the PX domain when the hydrophobic motif is in a phosphorylated state. We also examined the behavior of a kinase-dead mutant in the context of constitutively active SGK3, i.e. K191M SGK3-PRK2 (Fig. 8C). This mutant exhibits very low Thr-320 phosphorylation, indicating that SGK3 catalytically active conformation is important for its activation.

Taken together, our results point to an activation mechanism (Fig. 9) where the PX domain is responsible for localizing SGK3 in the endosomes, so that an endosomal staurosporine-sensitive kinase, in a PI3K-dependent manner, phosphorylates the SGK3 hydrophobic motif. PDK1, in a PI3K-dependent manner, docks to the phosphorylated HM of SGK3 and phosphorylates its activation loop residue, yielding fully active SGK3.

**DISCUSSION**

The PI3K pathway is frequently activated in human cancers. Much effort has focused on understanding the role of PKB/Akt in these processes, but there are other enzymes that are similarly PI3K-dependent, including SGK3. To begin to understand the contribution of this kinase to PI3K signaling, we have examined the mechanisms of activation of SGK3. The results reported here demonstrate the critical role of the PX domain in localization and subsequent phosphorylation and activation. By using phospho-selective antibodies, we monitored the phosphorylation of SGK3 on its activation loop (Thr-320) and its hydrophobic motif (HM) (Ser-486) and from these studies build a model of SGK3 regulation (Fig. 9).

We confirmed that human SGK3 contains a functional PX domain that majorly binds PI(3)P, reproducing the previous work (22). Despite the PLO being an in vitro assay, its result is consistent with the endosomal localization of SGK3. A crystal structure of the CISK PX domain suggests that its phospholipid binding pocket is wide enough to bind a PI with a highly phosphorylated head group (10). Our PLO also indicates that human
FIGURE 7. The activity of a fusion of SGK3 and the hydrophobic motif of PRK2 is independent of pervanadate and of PI3K. A, domain organization of SGK3-PRK2. B, HEK293 cells were transfected with FLAG-SGK3-PRK2 and treated with vehicle, 0.1 mM pervanadate or pretreated with 25 μM LY294002. Cell lysates were analyzed by immunoblot analysis using anti-phospho-Thr-320 SGK3 antibody. B, HEK293 cells were transfected with FLAG-SGK3 or FLAG-SGK3-PRK2 and treated as indicated. Kinase assays were performed on immunoprecipitates using Cross-tide as the peptide substrate. Error bars represent means ± S.E. Results shown are from three independent experiments. D, immunofluorescence was carried out as explained in Fig. 2 on Rat1 cells transfected with FLAG-SGK3-PRK2. E, COS7 cells were transfected with SGK3-PRK2 alone, HA-GSK3β alone, or both. Cell lysates were analyzed by immunoblot using anti-phospho-GSK3 antibody. DAPI, 4,6-diamidino-2-phenylindole. WT, wild type.
SGK3 can bind to PI(3,4)P_2 and PI(3,5)P_2. The SGK3 PX domain can accommodate larger PI, but we believe that it preferentially binds PI(3)P in its cellular context. Although PIP_3 is exquisitely regulated by PI3K agonists, the levels of the PI(3)P remain fairly constant within the cell. This monophosphate is the subject of constant turnover and is linked to housekeeping functions such as vesicle trafficking.

Contrary to PI(3,4,5)P_3, the product of PI3K type I, it is believed that PI3K type III enzymes are mostly responsible for the production of PI(3)P. Use of p85 knock-out embryonic fibroblasts revealed that class IA PI3K are not required for PI(3)P formation (23). In our hands, a GFP-PX probe for PI(3)P was only partially disrupted from the endosomes with LY294002 (data not shown), indicating that PI(3)P was not fully abolished by inhibiting the type I PI3K. We have shown that SGK3 is still localized to the endosomes with LY294002, indicating that SGK3 targeting to endosomes is independent of type I PI3K activation. We have observed that SGK3 is present in the endosomes without or with pervanadate treatment, suggesting again that an increase in PIP_3 levels does not alter SGK3 localization. A class II PI3K enzyme, a class known to associate with clathrin and to regulate endocytosis, termed PI3K with a C2 domain (PI3K-C2), was found to preferentially phosphorylate PI to generate PI(3)P, to contain a PX domain and to be refractory to wortmannin and LY294002 inhibition (24). It is therefore possible that PI3K-C2α is at play here. Therefore, the localization of SGK3 to endosomes is dependent on its PX domain and not on PI3K type I activity.

SGK3 localization, phosphorylation of Thr-320 and Ser-486, and protein kinase activity are dependent on the PX domain and the latter two properties on class I PI3K activity (from now on in this discussion, PI3K will refer to PI3K type I). An SGK3 mutant harboring the defective PX domain fails to bind PI(3)P, is not located at endosomes, and is not phosphorylated on either the activation loop or on the HM residue, suggesting that the specific localization of SGK3 to endosomes is a key requisite for activation. Furthermore, co-localization of SGK3 and PDK1 at the membrane is insufficient to trigger phosphorylation and activation of SGK3. Myristoylated version of CISK has been shown to be constitutively activated, but it is still localized to endosomes (5). Our results are also consistent with a dominant role for the PX domain in targeting CISK/SGK3. Given that the sequences of human and murine SGK3 are quite similar, we conclude that the constitutive activation of myr-CISK occurs because of a difference in the conformation of the murine gene product.

PDK1 phosphorylates Thr-320 on endosomally localized SGK3 can bind to PI(3,4)P_2 and PI(3,5)P_2. The SGK3 PX domain can accommodate larger PI, but we believe that it preferentially binds PI(3)P in its cellular context.

Although PIP_3 is exquisitely regulated by PI3K agonists, the levels of the PI(3)P remain fairly constant within the cell. This monophosphate is the subject of constant turnover and is linked to housekeeping functions such as vesicle trafficking. Contrary to PI(3,4,5)P_3, the product of PI3K type I, it is believed that PI3K type III enzymes are mostly responsible for the production of PI(3)P. Use of p85 knock-out embryonic fibroblasts
Regulation of SGK3 by Its PX Domain

FIGURE 9. Model of SGK3 mechanism of activation. Step 1, SGK3 is localized to the endosomes by its PX domain. An unidentified endosomal kinase activated by PI3K signals and sensitive to staurosporine phosphorylates the hydrophobic motif of SGK3. This phosphorylation event is dependent on the catalytic activity of SGK3. Step 2, PDK1 then binds to the phosphorylated hydrophobic motif and is then able to efficiently phosphorylate Thr-320. Step 3, the phosphorylated HM of SGK3, once liberated from PDK1, inserts itself into a PIF binding pocket within SGK3, thereby producing fully active SGK3.

SGK3. A remaining question is how PDK1 acquires its proximity to SGK3. It has been reported that PI3K activity is present in endosomes (25–28), and so it is conceivable, although unproven, that there exists a pool of endosomal PDK1. Endosomes are known to contain activated signaling complexes (29) and to both negatively regulate signaling cascades and to actively participate in carrying out cellular communication. In this case, it is possible that PI3K-activated receptors and membrane-bound PDK1 are internalized into endosomes and are thereby co-localized with SGK3. Such a model would suggest a temporal delay in PI3K-induced SGK3 activation, compared with activation of PKB at the plasma membrane, as well as a dependence upon receptor internalization and trafficking.

The importance of a phosphorylated HM is exemplified by the constitutive activation of SGK3-PRK2. Barford and co-workers (21) generated a fusion protein of PKBβ and the HM of PRK2. This crystal structure revealed that the role of the HM is in promoting intramolecular binding of this domain to the amino-terminal lobe of the kinase domain. This binding promotes a disorder-to-order transition of the αC helix, which then interacts with phosphorylated Thr-309, undergoes restructuring, and orders the activation segment to yield a fully active kinase. These authors found that PKBβ PRK2 was a much better substrate for PDK1 (and for itself) than PKBβ with its natural HM (16). Our SGK3-PRK2 chimeric protein also seems to adopt a stable conformation that results in constitutive activation. Hence, this mutant highlights the significance of complementing the SGK3 catalytic core by hydrophobic interactions with PDK1 and then, with itself, to produce a fully active kinase.

To understand further the relative contribution of the HM and the PX domain in SGK3 activation, we mutated the PX domain in SGK3-PRK2 to inactivate it. Contrary to wild type SGK3, the PX domain is no longer required to generate a phosphorylated and active form of SGK3 when the HM is phosphorylated (or in this case, a mimic of a phosphorylated HM), suggesting that one important role of the PX domain is to direct SGK3 to endosomes where it is phosphorylated on its HM. In light of the finding that the kinase-inactivated SGK3 mutant retains endosomal targeting but is neither phosphorylated nor active, there must be additional inputs for phosphorylation. The lack of phosphorylation of kinase-dead SGK3 and of kinase-dead SGK3 in the context of constitutively active SGK3 hints that an active conformation may play a role in its mechanism of activation. Although our results indicate that localization to endosomes is important for HM phosphorylation, it is possible that the SGK3 HM kinase mentioned here does not constitutively reside in the endosomes. The HM kinase may be recruited to endosomes, maybe in response to PI3K signals. The potential candidates for the HM kinase of PKB, such as PKCβII, ATM, and mTOR, should be tested for SGK3 activation.

Because the SGK family of kinases is structurally most similar to the PKB family, it is useful to compare their regulation. The first obvious difference is the nature of their phospholipid binding domains, which localize both proteins to different cell compartments. PKB localization is transient and only occurs in the few minutes when production of 3′-phosphorylated lipids occurs. SGK3 remains in the endosomal compartment once activated, unlike phosphorylated PKB, which diffuses from the plasma membrane into the cytoplasm and nucleus (30). The constitutive SGK3 endosomal localization allows it to be in close proximity to its HM kinase when SGK3-inducing signals occur, whereas PKB membrane targeting is important for co-localizing it with PDK1 as a consequence of its mutual attraction to 3′-phosphorylated lipids via respective pleckstrin homology domains. Also, our data support the fact that SGK3, much like SGK1 (17), relies more heavily than PKB on docking motif interactions with PDK1 for activation. Another potential distinction is the nature of the HM kinase. Kinase-dead SGK3 was not phosphorylated on Ser-486, and Ser-486 phosphorylation was found to be sensitive to staurosporine. In contrast, staurosporine does not inhibit Ser-473 phosphorylation of PKB (31). We therefore speculate that the identity of the kinases that target the hydrophobic motifs of PKB and SGK3 may be different, based on their cellular location.
A key issue is the mechanism by which SGK3 phosphorylation/activation is dependent upon PI3K. PDK1 activity toward SGK3 does not appear to be affected by this signal, and the dependence can be overcome by mimicking phosphorylation of the HM. This points to the HM kinase as the PI3K-dependent component of the system. If SGK3 autophosphorylation does play a part in this function, it is not clear how this activity is PI3K-sensitive. We were only able to achieve SGK3 activation with pervanadate, suggesting that PI3K activation is necessary but not sufficient for SGK3 activation. It is plausible that the PI3K in combination with other inputs are needed to fully activate this HM kinase. We have preliminary data indicating that SGK3 is responsive to cyclic AMP. Another possible input is inactivation of myotubularins, a family of lipid phosphatases that dephosphorylate PI(3)P and are inhibited by pervanadate. We have preliminary data indicating that PI3K-sensitive. We were only able to achieve SGK3 activation with pervanadate, suggesting that PI3K activation is necessary but not sufficient for SGK3 activation. It is plausible that the PI3K in combination with other inputs are needed to fully activate this HM kinase. We have preliminary data indicating that SGK3 is responsive to cyclic AMP. Another possible input is inactivation of myotubularins, a family of lipid phosphatases that dephosphorylate PI(3)P and are inhibited by pervanadate (32).

The differential subcellular targeting of SGK3 and PKB also implies differences in their substrate specificity and physiological functions. PKB is the subject of considerable effort as a therapeutic target because of its hyperactivation in several human diseases. Consequently, determining the physiological relevance of SGK3 to these processes may promote this molecule as a new drug target. The generation of a constitutively activated mutant of SGK3 will allow generation of transgenic models to assess the effects of activation of this protein kinase in vivo.

Acknowledgments—We thank Michael P. Scheid and Bradley W. Doble for scientific advice.

REFERENCES

1. Webster, M. K., Goya, L., Ge, Y., Maiyar, A. C., and Firestone, G. L. (1993) Mol. Cell. Biol. 13, 2031–2040
2. Kobayashi, T., and Cohen, P. (1999) Biochem. J. 339, 319–328
3. Kobayashi, T., Deak, M., Morrice, N., and Cohen, P. (1999) Biochem. J. 344, 189–197
4. Park, J., Leong, M. L., Buse, P., Maiyar, A. C., Firestone, G. L., and Hemmings, B. A. (1999) EMBO J. 18, 3024–3033
5. Liu, D., Yang, X., and Songyang, Z. (2000) Curr. Biol. 10, 1233–1236
6. Sato, T. K., Overduin, M., and Emr, S. D. (2001) Science 294, 1881–1885
7. Gillooly, D. J., Morrow, I. C., Lindsay, M., Gould, R., Bryant, N. J., Gaultier, J. M., Parton, R. G., and Stenmark, H. (2000) EMBO J. 19, 4577–4588
8. Geiser, M., Cebe, R., Drewello, D., and Schmitz, R. (2001) BioTechniques 31, 88-90, 92
9. Dowler, S., Kular, G., and Alessi, D. R. (2002) Sci. STKE 2002, PL6
10. Xing, Y., Liu, D., Zhang, R., Joachimiak, A., Songyang, Z., and Xu, W. (2004) J. Biol. Chem. 279, 39662–39669
11. Gillooly, D. J., Raiborg, C., and Stenmark, H. (2003) Histochem. Cell Biol. 120, 445–453
12. Firestone, G. L., Giamp Paolo, J. R., and O’Keeffe, B. A. (2003) Cell. Physiol. Biochem. 13, 1–12
13. Scheid, M. P., Marignani, P. A., and Woodgett, J. R. (2002) Mol. Cell. Biol. 22, 6247–6260
14. Staal, S. P., Hartley, J. W., and Rowe, W. P. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3065–3067
15. Resh, M. D. (1999) Biochim. Biophys. Acta 1451, 1–16
16. Biondi, R. M., Cheung, P. C., Casamayor, A., Deak, M., Currie, R. A., and Alessi, D. R. (2000) EMBO J. 19, 979–988
17. Biondi, R. M., Kieloch, A., Currie, R. A., Deak, M., and Alessi, D. R. (2001) EMBO J. 20, 4380–4390
18. Ruegg, U. T., and Burgess, G. M. (1989) Trends Pharmacol. Sci. 10, 218–220
19. Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P., and Alessi, D. R. (1999) Curr. Biol. 9, 393–404
20. Yang, J., Cron, P., Thompson, V., Good, V. M., Hess, D., Hemmings, B. A., and Barford, D. (2002) Mol. Cell 9, 1227–1240
21. Yang, J., Cron, P., Good, V. M., Thompson, V., Hemmings, B. A., and Barford, D. (2002) Nat. Struct. Biol. 9, 940–944
22. Virbasius, J. V., Song, X., Pomerleau, D. P., Zhan, Y., Zhou, G. W., and Czech, M. P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12908–12913
23. Vieira, O. V., Botelho, R. J., Rameh, L., Brachmann, S. M., Matsu, T., Davidson, H. W., Schreiber, A., Backer, J. M., Cantley, L. C., and Grinstein, S. (2001) J. Cell Biol. 155, 19–25
24. Domin, J., Pages, F., Volinia, S., Rittenhouse, S. E., Zvelebil, M. J., Stein, R. C., and Waterfield, M. D. (1997) Biochem. J. 326, 139–147
25. Shpetner, H., Joly, M., Hartley, D., and Corvera, S. (1996) J. Cell Biol. 132, 595–605
26. Li, G., D’Souza-Schorey, C., Barbieri, M. A., Roberts, R. L., Klippel, A., Williams, L. T., and Stahl, P. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10207–10211
27. Jones, A. T., and Clague, M. J. (1995) Biochem. J. 311, 31–34
28. Spiro, D. J., Boll, W., Kirchhausen, T., and Wessling-Resnick, M. (1996) Mol. Biol. Cell 7, 355–367
29. Sorkin, A., and Von Zastrow, M. (2002) Nat. Rev. Mol. Cell Biol. 3, 600–614
30. Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 31515–31524
31. Hill, M. M., Andjelkovic, M., Brazil, D. P., Ferrari, S., Fabbro, D., and Hemmings, B. A. (2001) J. Biol. Chem. 276, 25643–25646
32. Clague, M. J., and Lorenzo, O. (2005) Traffic 6, 1063–1069