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Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway

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Serum and glucocorticoid-inducible kinase (SGK) is a novel member of the serine/threonine protein kinase family that is transcriptionally regulated. In this study, we have investigated the regulatory mechanisms that control SGK activity. We have established a peptide kinase assay for SGK and present evidence demonstrating that SGK is a component of the phosphoinositide 3 (PI 3)-kinase signaling pathway. Treatment of human embryo kidney 293 cells with insulin, IGF-1 or pervanadate induced a 3- to 12-fold activation of ectopically expressed SGK. Activation was completely abolished by pretreatment of cells with the PI 3-kinase inhibitor, LY294002. Treatment of activated SGK with protein phosphatase 2A *in vitro* led to kinase inactivation. Consistent with the similarity of SGK to other second-messenger regulated kinases, mutation of putative phosphorylation sites at Thr256 and Ser422 inhibited SGK activation. Cotransfection of PDK1 with SGK caused a 6-fold activation of SGK activity, whereas kinase-dead PDK1 caused no activation. GST-pulldown assays revealed a direct interaction between PDK1 and the catalytic domain of SGK. Treatment of rat mammary tumor cells with serum caused hyperphosphorylation of endogenous SGK, and promoted translocation to the nucleus. Both hyperphosphorylation and nuclear translocation could be inhibited by wortmannin, but not by rapamycin.

Keywords: insulin/phosphoinositide 3-kinase/3-phosphoinositide-dependent protein kinase 1/SGK protein kinase/signal transduction

Introduction

A diverse set of environmental cues utilize intracellular protein phosphorylation–dephosphorylation cascades to rapidly and reversibly transduce their signals from their plasma membrane receptors to the cytoplasm and the nucleus. The regulation of individual protein components within these cascades provides the biological specificity and flexibility that allows cells to respond quickly to extracellular stimuli in a physiologically appropriate manner. The serum and glucocorticoid-inducible kinase (SGK) is a novel member of the serine/threonine protein kinase gene family that was first identified from the Con8.hd6 rat mammary tumor cell line (Webster *et al.*, 1993a). In contrast to most characterized protein kinases, an unusual property of SGK is its acute transcriptional control by several distinct signal transduction pathways. The SGK promoter contains a functional glucocorticoid response element, which accounts for its glucocorticoid inducibility (Maiyar *et al*., 1997), and a SP-1 regulatory element that mediates its induction by follicle stimulating hormone/forskolin in ovarian cells (Alliston *et al.*, 1997), and is a transcriptional target of the p53 tumor suppressor protein (Maiyar *et al.*, 1996). The stimulated transcription of SGK is also an immediate early response to serum (Webster *et al.*, 1993b). Depending on the tissue type, SGK expression has been shown to be induced by injury or cell volume changes, and more recently by aldosterone in kidney cells (Imaizumi *et al.*, 1994; Hollister *et al.*, 1997; Waldegger *et al*., 1997; Chen *et al*., 1999), or suppressed by heparin which is known to inhibit vascular smooth muscle cell proliferation (Delmolino and Castellot, 1997). It is likely that these responses also involve the regulation of SGK promoter activity. SGK can also be regulated at the post-translational level because the cytoplasmic–nuclear shuttling of this protein has recently been shown to proceed in synchrony with the cell cycle (Buse *et al.*, 1999). Thus, SGK is probably a functional convergence point between several types of cell signaling pathways and cellular phosphorylation cascades. However, the precise role of SGK in the coordination of these processes is only poorly understood because the regulation of its kinase activity has not been studied previously.

SGK displays similarity (45–55% sequence identity) throughout its catalytic domain with protein kinase B (PKB/cAKT), protein kinase C, ribosomal protein S6 kinase and cyclic AMP-dependent protein kinase (Webster *et al*., 1993a) (see Figure 1). Recently, it was reported that the activation loop (A-loop) site in the second-messenger regulated protein kinase family is phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1), first identified as one of the upstream kinases activating PKB (Alessi *et al*., 1997a; Stokoe *et al.*, 1997). Thus, PDK1 phosphorylates PKB, which is implicated in glucose metabolism, transcriptional control and in the regulation of apoptosis in many different cell types (reviewed in Galetić *et al*., 1999), within the A-loop on Thr308 (Alessi *et al*., 1997b; Stokoe *et al.*, 1997). PDK1 has also been shown to phosphorylate $p70^{S6K}$ (Alessi *et al*., 1998; Pullen *et al*., 1998), a key molecule in the control of protein synthesis, at the analogous A-loop site, Thr229. Further studies have demonstrated PDK1 phosphorylation at Thr197 in protein kinase A (PKA) (Cheng *et al*., 1998) and Thr410 in PKCζ (Chou *et al*., 1998; Le Good *et al*., 1998).

Here we report the development of a kinase assay

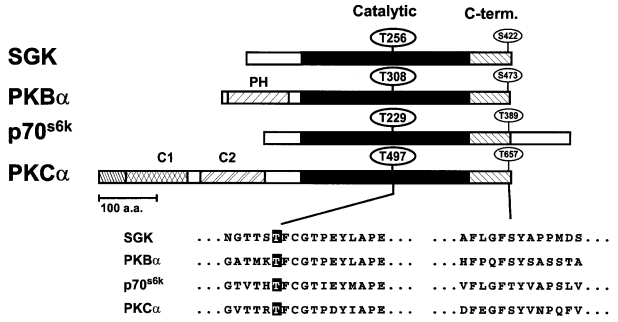

Fig. 1. Schematic presentation of SGK structure and comparison with other second-messenger regulated kinases. Putative sites of SGK phosphorylation within the activation loop, Thr256, and in the C-terminus, Ser422, are compared with analogous sites in other second-messenger regulated kinases. Threonine residues known to be phosphorylated by PDK1 are indicated (highlighted). Amino acid sequences surrounding sites of phosphorylation in the various kinases are shown below. PH, pleckstrin homology domain; C1, diacylglycerol/phorbol ester binding domain; C2, Ca^{2+}/TPA binding domain.

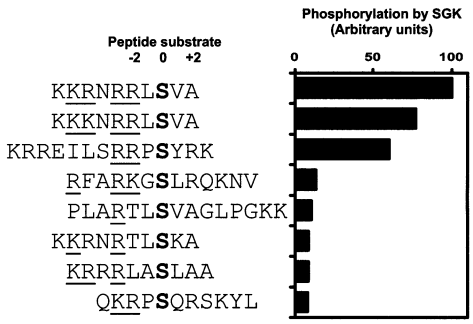

Fig. 2. Development of an SGK protein kinase assay. Immunoprecipitated HA-SGK was prepared from HEK 293 cells. Peptides were tested for phosphorylation by HA-SGK as described in Materials and methods. The extent of phosphorylation of each peptide is expressed as a percentage relative to the optimal substrate peptide, Sgktide (KKRNRRLSVA). The serine phosphoacceptor (position 0) is shown in bold.

permitting biochemical analysis of SGK regulation, function and substrate specificity. We demonstrate that SGK is a downstream target of phosphoinositide 3-kinase (PI 3-kinase)-stimulated growth factor signaling, and that PDK1 can phosphorylate the A-loop site of SGK *in vivo* and *in vitro*, leading to activation of SGK.

Results

Establishment of an SGK protein kinase assay

SGK has been shown to be transcriptionally regulated by several specific hormonal and environmental signals. However, until now, studies demonstrating enzyme activity for SGK have been unsuccessful because SGK fails to transphosphorylate traditional substrates, such as histone H1, myelin basic protein and casein (Webster *et al*., 1993a). To develop a novel *in vitro* kinase assay for SGK, we prepared lysates from human embryo kidney 293 (HEK 293) cells expressing wild-type hemagglutinin (HA)-tagged SGK, and screened a library composed of synthetic peptides (sequences available upon request) for a potential SGK substrate. Several peptides were identified as SGK substrates, and a selection of these are shown in Figure 2. Of the peptides tested, the best substrate for SGK was KKRNRRLSVA, derived from the optimal peptide substrate for p90 ribosomal protein S6 kinase (Leighton *et al.*, 1995). This peptide was named Sgktide. By comparing the phosphorylation of Sgktide with the other peptides, we concluded that the arginines at the –2/–3 and –5/–6 positions are required for SGK activity. In addition, basic residues on the C-terminal side of the phosphorylation site appear to be inhibitory. In subsequent experiments, Sgktide was used as an SGK substrate and basic parameters for the kinase assay were established (immunoprecipitation of HA-tagged, wild-type SGK from 400 μg of cell extracts and 60 min of incubation for the kinase assay). Similar results in terms of substrate specificity were obtained using HA-SGK from pervanadate-treated HEK 293 cells (see below).

HEK 293 cells were used to investigate the possible involvement of SGK in the PI 3-kinase signaling pathway. Immunoprecipitated HA-tagged SGK activity was increased ~13-fold by treating cells with pervanadate, which is a well characterized inhibitor of tyrosine phosphatases and mimics insulin signaling (Posner *et al*., 1994; Andjelković *et al*., 1996). In contrast, HA-SGK activation was completely abolished by pretreatment of cells with the specific inhibitor of PI 3-kinase, LY294002. Furthermore, transfection of a kinase-dead form of SGK with a methionine substitution at lysine 127 of the ATP binding site failed to phosphorylate the Sgktide substrate (Figure 3A). Thus, SGK is responsible for all of the activity present in the HA-SGK immunoprecipitates. The HA-SGK protein from untreated cells migrated as a doublet during SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot analysis (Figure 3A, lane 1). However, a shift in electrophoretic mobility is seen following pervanadate treatment probably due to a change in phosphorylation state. These slower migrating forms of SGK were previously shown to be phosphorylated by their incorporation of ^{32}P-phosphate (Buse *et al.*, 1999). The mobility shift in pervanadate-treated cells was also observed for the kinase-deficient form, HA-SGK-K127M, even though kinase activity was undetectable (Figure 3A, lower panel).

Activation occurred within 5 min, kinase activity remained high for at least 15 min (Figure 3B) and correlated with decreased mobility of HA-SGK on SDS–PAGE. These results suggested that SGK is a component of the PI 3-kinase signaling pathway and SGK activity is modulated by reversible phosphorylation.

In order to investigate this possible regulation by phosphorylation, we tested the *in vivo* effects of a serine/threonine protein phosphatase inhibitor on HA-SGK from HEK 293 cells. Strikingly, treatment of the cells with calyculin-A, a specific inhibitor of protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1), induced an ~17-fold increase in HA-SGK activity and concomitant change in electrophoretic mobility, as expected for the hyperphosphorylated form of SGK (Figure 3C, lane 4). The addition of H_2O_2 (0.2 mM) or 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (100 ng/ml) to transfected cells did not lead to significant increase of HA-SGK activity (Figure 3C). However, it should be noted that higher concentrations of H_2O_2 (5 mM) were found to promote robust kinase activation (data not shown).

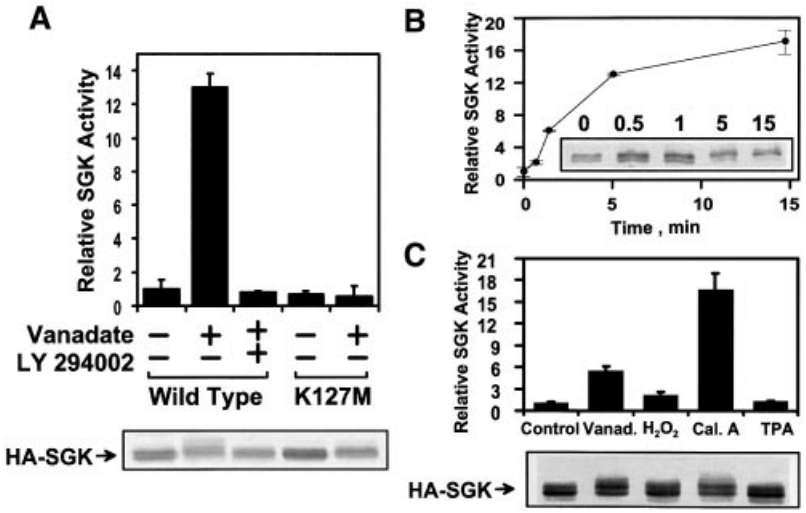

Fig. 3. Regulation of exogenous SGK activity by pervanadate through the PI 3-kinase pathway in HEK 293 cells. (**A**) HEK 293 cells overexpressing SGK were either treated with 0.1 mM pervanadate for 15 min or pretreated with 50 μM LY 294002 for 15 min, followed by 0.1 mM pervanadate for 15 min. HA-SGK was immunoprecipitated from cell extracts with 12CA5 monoclonal and the immune complexes were assayed for kinase activity using Sgktide as substrate. Kinase activity is the average ± standard deviation (SD) of three independent experiments. Activity of SGK immunoprecipitated from quiescent cells was taken as 1. SGK migration following the above treatments and detected by immunoblot analysis is shown (lower panel). (**B**) Time course of SGK activation following pervanadate stimulation of HEK 293 cells. Kinase activity is a typical result of three independent experiments. Activity of SGK immunoprecipitated from unstimulated cells was taken as 1. Immunoblot analysis of SGK (insert). (**C**) HEK 293 cells overexpressing SGK were treated with 0.1 mM pervanadate, 0.2 mM H_2O_2, 100 nM calyculin-A or 100 ng/ml TPA for 10 min. Immunoprecipitated HA-SGK was assayed for kinase activity using Sgktide as substrate. Kinase activity is the average (±SD) of three experiments with duplicate immunoprecipitates. Activity of SGK immunoprecipitated from untreated cells was taken as 1. SGK immunoblot analysis is shown in the lower panel.

SGK activity is regulated by reversible protein phosphorylation

The mobility shift detected by SDS–PAGE during pervanadate treatment of cells and during activation by calyculin-A suggested that SGK activity is modulated by reversible protein phosphorylation. To test this possibility, we investigated the *in vitro* effects of the PP2A on HA-SGK kinase immunoprecipitated from pervanadate-stimulated HEK 293 cells. Immunoprecipitated HA-SGK was incubated with PP2A catalytic subunit purified from the baculovirus system (T.Myles and B.A.Hemmings, unpublished data). Dephosphorylation of the activated SGK *in vitro* by PP2A resulted in an ~96% reduction of kinase activity and corresponding increase in electrophoretic mobility (Figure 4). Inhibition of PP2A *in vitro* by 100 nM okadaic acid (OA) prevented SGK inactivation and the increase in electrophoretic mobility. These results confirm that SGK activity is regulated by reversible phosphorylation.

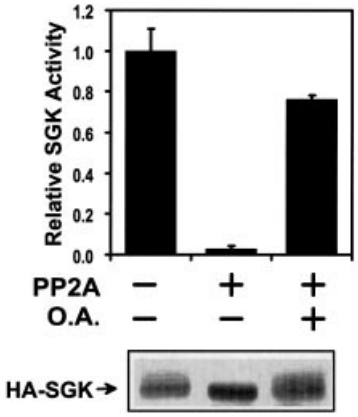

Fig. 4. Inactivation of SGK by PP2Ac *in vitro*. Cells were stimulated with 100 μM pervanadate for 15 min and HA-SGK was immunoprecipitated from extracts. The immune complexes were incubated with PP2Ac in the presence or absence of okadaic acid or buffer alone for 30 min, then SGK activity was measured with Sgktide as substrate. Relative kinase activity is the average (±SD) of two experiments with duplicate immunoprecipitates. Activity of stimulated SGK was taken as 1.

Insulin and IGF-1 promote SGK activation

PI 3-kinase has been implicated in several cellular responses resulting from the stimulation of growth factor receptors, including the promotion of cell survival, cytoskeletal rearrangements and vesicular trafficking (Carpenter and Cantley, 1996). Also, SGK mRNA synthesis is known to be stimulated by glucocorticoids and serum within 30 min (Webster *et al.*, 1993a). To address whether SGK is involved in insulin or IGF-1 signaling, we examined the effects of these agonists on SGK activity *in vivo*. Treatment of HEK 293 cells with insulin or IGF-1 induced a 4.5- or 3.5-fold increase in HA-SGK activity, respectively, and an electrophoretic mobility shift similar to that observed during pervanadate treatment of cells (Figure 5). This activation of HA-SGK and concomitant gel shift promoted by insulin or IGF-1 was inhibited by pretreatment of cells with LY294002. These results indicate that SGK is a downstream target of the insulin/IGF-1 receptors with activation being mediated by lipid second messengers generated by PI 3-kinase.

To test further whether SGK activation is dependent on PI 3-kinase, HA-SGK was cotransfected with a constitutively activated, membrane-targeted form of PI 3-kinase (p110-CAAX) (Didichenko *et al.*, 1996). Transient transfection of HEK 293 cells followed by kinase assays revealed that cotransfection of SGK with wild-type p110-CAAX caused an 8-fold activation of HA-SGK accom-

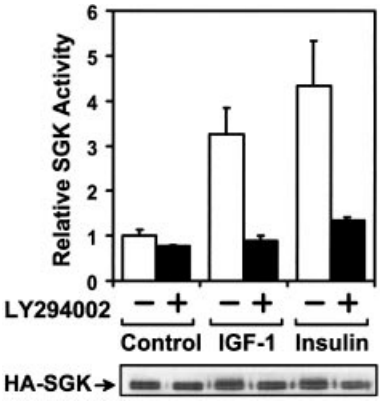

Fig. 5. The effects of insulin and IGF-1 on SGK activation. HEK 293 cells transiently transfected with pCMV4. HA-SGK were treated for 15 min with 50 μM LY294002 or drug vehicle prior to 15 min stimulation with either 50 ng/ml IGF-1 or 100 nM insulin. HA-SGK was immunoprecipitated from cell extracts with the 12CA5 antibody and the immune complexes were assayed for kinase activity. Kinase activity is a typical result of six independent experiments with duplicate immunoprecipitates. SGK activity immunoprecipitated from untreated cells was taken as 1. The expression level of HA-SGK in HEK 293 cells was determined by immunoblot analysis using the 12CA5 monoclonal antibody (lower panel).

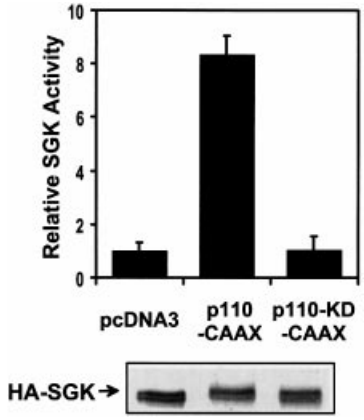

Fig. 6. Effect of membrane-targeted PI 3-kinase on SGK activity. Wild-type HA-p110-CAAX, kinase-deficient HA-p110-KD-CAAX or pcDNA3 vector were transiently cotransfected into HEK 293 cells with HA-SGK. HA-SGK was immunoprecipitated from the extracts with 12CA5 antibody, and immunoprecipitated HA-SGK activity was assayed in the presence of LY294002 to inhibit the PI 3-kinase activity using Sgktide as substrate. Kinase activity is the average (±SD) of three experiments with duplicate immunoprecipitates. Activity of SGK immunoprecipitated from pcDNA3-vector transfected cells was taken as 1. The expression from each construct was observed to be equal by immunoblot analysis (lower panel).

panied by mobility shift (Figure 6). However, a kinase-deficient p110-CAAX control caused no activation or mobility shift for immunoprecipitated HA-SGK (Figure 6). These results provide further evidence that SGK is downstream of PI 3-kinase.

Mutation of the putative phosphorylation sites of SGK abolishes its activation in HEK 293 cells

According to the sequence alignment of SGK with other second-messenger regulated kinases (Figure 1), putative regulatory phosphorylation sites in SGK are Thr256 in the A-loop and Ser422 in the C-terminal domain. In view of the fact that SGK is regulated in a manner very similar to PKB, we decided to investigate the role of these

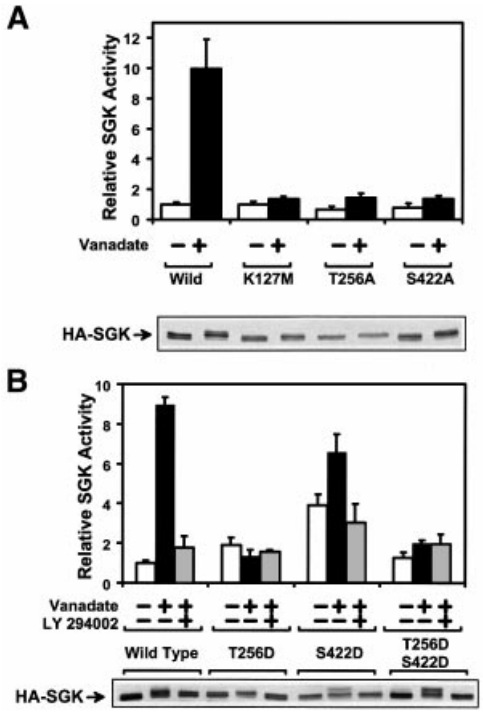

Fig. 7. Effect of SGK phosphorylation mutants on activation by pervanadate. HEK 293 cells were transiently transfected with wild-type HA-SGK or the mutants [(**A**) HA-SGK K127M, HA-SGK T256A, HA-SGK S422A, (**B**) HA-SGK T256D, HA-SGK S422D or HA-SGK DD]. Cells were pretreated with (B) or without (A) 50 μM LY294002 for 15 min followed by 15 min treatment with 0.1 mM pervanadate or buffer. Immunoprecipitated SGK from the extracts was assayed for kinase activity, after correcting SGK expression levels for each DNA construct. The results are expressed as fold activation relative to the specific activity of wild-type HA-SGK from unstimulated HEK 293 cells. Equal amounts of extracts were immunoblotted with 12CA5 monoclonal to monitor expression levels of different SGK mutants in HEK 293 cells (lower panel).

sites in kinase regulation by carrying out an extensive mutational analysis of these sites. Therefore, Thr256 and Ser422 were mutated to Ala or Asp to investigate the role of these sites in kinase regulation. The results of this analysis are shown in Figure 7. For these experiments we stimulated the cells with pervanadate (prepared according to the methods of Posner *et al*., 1994) because it is the most potent activator of SGK activity. We have obtained similar results using insulin and IGF-1 (data not shown). As found earlier (cf. Figure 3A), HA-SGK activity is elevated 10-fold by pervanadate treatment, concomitant with a change in electrophoretic mobility (Figure 7A). The kinase-dead HA-SGK K127M also shows the same mobility shift without increase in activity. Conversion of Thr256 to Ala almost completely blocked kinase activation, as did the mutation of Ser422 to Ala (Figure 7A). Significantly, the single phosphorylation site mutants still showed a mobility shift on SDS–PAGE, suggesting that the SGK protein is being phosphorylated at other phosphorylation sites. In the case of PKB, a single phosphorylation site mutation (Thr308 to Ala or Ser473 to Ala) did not block the phosphorylation of the second regulation site (Alessi *et al*., 1996), indicating independent regulation of these two sites. From this analysis we conclude that both Thr256 and Ser422 are required for complete activation of SGK. At this juncture

we cannot exclude that other sites in SGK are constitutively phosphorylated (modified in unstimulated cells) or become phosphorylated following stimulation of cells. Significantly, there are several Ser/ThrPro (S/TP) sites in the N- and C-terminal regions of the kinase outside of the catalytic domain.

In order to extend these studies, we mutated the Thr256 and Ser422 to an acid residue (Asp) to mimic the effect of phosphorylation (Figure 7B). These mutant derivatives of SGK were analyzed in the HEK 293 cells using pervanadate stimulation. Mutation of Thr256 to Asp led to a modest activation of kinase in unstimulated cells (found in two experiments), but apparently blocked further activation following pervanadate treatment. Significantly, the migration of this mutant in SDS–PAGE was also decreased, and this was inhibited by LY294002, suggesting that phosphorylation of Ser422 takes place following pervanadate treatment. Conversion of Ser422 to Asp led to a significant increase (8-fold) of kinase activity that could be enhanced with pervanadate treatment (Figure 7B) concomitant with a dramatic reduction of electrophoretic mobility, suggesting that Thr256 becomes phosphorylated during activation. Contrary to our expectations, the double acidic mutant (T256D/S422D) is not constitutively active. In the case of PKB, conversion of the two major regulatory sites (Thr308 and Ser 473) to acidic residues converts the kinase to a partially constitutively active form (Alessi *et al*., 1996). At present we do not have an explanation for the behavior of SGK. Unexpectedly, we find that the migration of this double acidic mutant on SDS–PAGE following pervanadate treatment is decreased (Figure 7B). This result indicates that other sites in SGK are also phosphorylated during cell stimulation. Mutation of Ser78 (an SP site) to a non-phosphorylated residue suggest that this is a phosphorylation site during serum stimulation in Con8.hd6 cells (data not shown). Taken together, our results show that Thr256 and Ser422 are important regulatory phosphorylation sites in SGK, and other sites in this kinase are also modified during stimulation of HEK 293 cells. Currently, we are mapping the phosphorylation sites using mass spectrometry to obtain a complete picture of kinase modification during cell stimulation.

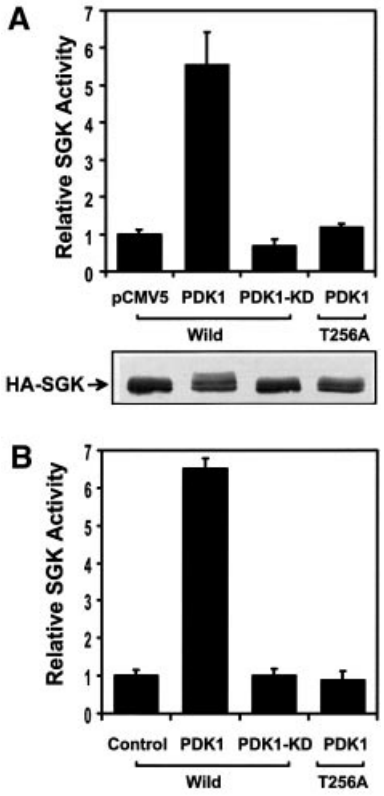

Fig. 8. PDK1 activates SGK *in vivo* and *in vitro*. (**A**) Wild-type Myc-PDK1, kinase-deficient Myc-PDK1-KD or pCMV5 vector constructs were transiently cotransfected with HA-SGK or HA-SGK T256A into HEK 293 cells. Immunoprecipitated HA-SGK and HA-SGK T256A were assayed for kinase activity using Sgktide. Kinase activity is the average (±SD) of three experiments with duplicate immunoprecipitates. SGK activity immunoprecipitated from pCMV5-vector transfected cells was taken as 1. Expression of each construct was equal as determined by immunoblot analysis (lower panel). (**B**) HA-SGK (control), HA-SGK T256A, Myc-PDK1 or Myc-PDK1-KD were independently expressed in HEK 293 cells. Extracts from cells expressing all four were immunoprecipitated with monoclonal anti-HA (12CA5) or anti-Myc (9E10) antibodies. HA-SGK and HA-SGK T256A were eluted from immune complexes with HA epitope peptide in kinase buffer, and a coupled kinase assay was performed as described in the Materials and methods. Kinase activity is the average (±SD) of three experiments with duplicate eluted HA-SGK. Activity of SGK from buffer-treated cells was taken as 1.

In vivo activation and in vitro phosphorylation of SGK by PDK1

Phosphorylation appears to be a regulatory mechanism for SGK activation. SGK contains putative phosphorylation sites conserved among other members of second-messenger regulated protein kinases (Figure 1). One of these sites is Thr256, analogous to Thr308 of PKB, and is phosphorylated by PDK1). We therefore tested if human PDK1 (Pullen *et al*., 1998) phosphorylates Thr256 of SGK and activates the kinase. For *in vivo* experiments, wild-type Myc-tagged PDK1 or kinase-deficient Myc-PDK1-KD constructs were transiently transfected into HEK 293 cells and the effect on coexpressed HA-SGK or HA-SGK-T256A activity was determined. Kinase assays revealed that cotransfection with wild-type PDK1 caused a 6-fold activation of SGK, whereas kinase-dead PDK1 (PDK1-KD) had no effect (Figure 8A). In addition, HA-SGK T256A activity was not affected by coexpressed PDK1. These results indicate that Thr256 in the A-loop of SGK is the target site for activation by PDK1. Moreover, in this coexpression experiment, the mobility shift of SGK occurred concomitantly with the change in SGK activation. As a control experiment, HA-tagged PKB lacking the PH domain (ΔPH-PKBα), which is a substrate for PDK1 in the absence of phospholipids (Alessi *et al*., 1997b), was cotransfected with PDK1 or PDK1-KD and effects on activity were determined. A 28-fold activation of ΔPH-PKBα was observed following the cotransfection with PDK1 (data not shown).

For the *in vitro* experiments, HA-SGK, HA-SGK T256A, Myc-PDK1 or Myc-PDK1-KD were independently expressed in HEK 293 cells. Extracts from cells expressing HA-SGK, HA-SGK-T256A, Myc-PDK1 or Myc-PDK1-KD were immunoprecipitated with monoclonal anti-HA (12CA5) or anti-Myc (9E10) antibodies, respectively. HA-SGK and HA-SGK T256A were eluted from immune complexes with HA epitope peptide in kinase buffer, and a coupled-kinase assay was used to determine the activity of eluted HA-SGK activity in the

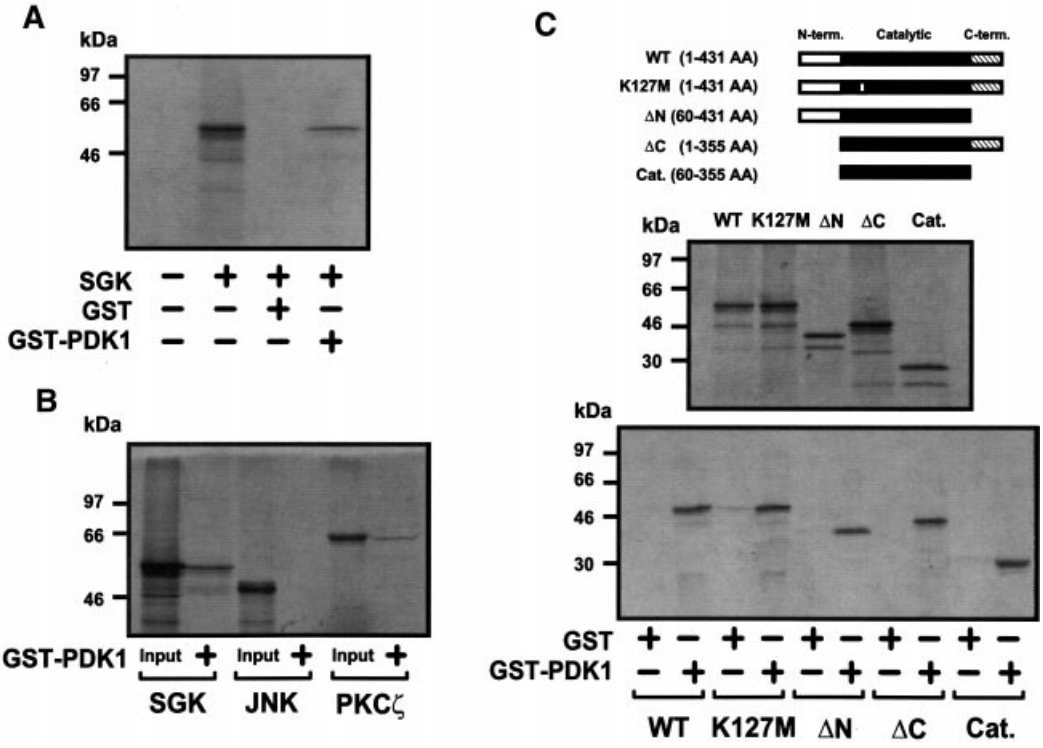

Fig. 9. Interaction between PDK1 and SGK *in vitro*. (**A**) cDNA for full-length wild-type SGK was transcribed and translated *in vitro*. The [^{35}S]methionine-labeled *in vitro* translation (IVT) product was incubated with GST alone (lane 3) or GST–PDK1 (lane 4), as indicated. Following recovery of fusion protein on glutathione–Sepharose beads, the bound fraction was analyzed by SDS–PAGE and visualized by autoradiography. The unprogrammed lysate is shown in lane 1, and 10% of the IVT product is represented in lane 2. (**B**) The IVT product of either full-length SGK, JNK or PKCζ was incubated with GST–PDK1 (lanes 2, 4 and 6) and proteins bound to the beads resolved by SDS–PAGE as described in (A). The IVT designated as input in lanes 1, 3 and 5 show 10% of the labeled proteins used in the binding assays. (**C**) Various fragments of SGK comprised of wild-type full-length (WT-SGK,1–431 aa), kinase-dead (K127M-SGK, 1–431 aa), N-terminal deleted SGK (ΔN-SGK, 60–431 aa), C-terminal deleted SGK (ΔC-SGK, 1–355 aa) and catalytic domain only SGK (Cat.-SGK, 60–355 aa) were synthesized as [^{35}S]methionine-labeled products as in (A), and incubated with either GST alone (lower panel, lanes 1, 3, 5, 7 and 9) or GST–PDK1 (lanes 2, 4, 6, 8 and 10) and samples processed as in (A). The lanes spanning 1–5 (middle panel) depict 10% of the IVT products included in the binding reactions. Molecular weight markers are shown on the far left side of each panel in (A), (B) and (C). Similar results were obtained in three separate experiments.

presence or absence of PDK1, as described in the Materials and methods. The HA-SGK activity was increased 7-fold upon incubation with PDK1, but activity of HA-SGK T256A was unaffected. Moreover, HA-SGK activity was not affected in the presence of Myc-PDK1-KD (Figure 8B). In parallel, the reactions were performed with [γ-^{32}P]ATP to monitor the phosphorylation of HA-SGK by PDK1. Consistent with *in vivo* activation of SGK by PDK1, the incubation of Myc-PDK1 with HA-SGK led to a robust increase in the amount of phosphate incorporated into HA-SGK; the signal was not detectable with HA-SGK T256A (data not shown).

In vitro association of PDK1 with SGK

In order to confirm the interaction of between PDK1 and SGK, *in vitro* glutathione *S*-transferase (GST)-pull down assays were performed. PDK1 was expressed as a GST fusion protein and tested for its ability to bind specifically to [^{35}S]methionine-labeled *in vitro* translation product of full-length SGK. Incubation of GST–PDK1 with [^{35}S]methionine-labeled SGK followed by recovery of bound SGK on glutathione–Sepharose beads revealed that GST–PDK1 and SGK associate *in vitro* (Figure 9A, lane 4), whereas in contrast, SGK displayed negligible binding with GST alone (Figure 9A, lane 3). To evaluate the specificity of interaction between SGK and PDK1, the GST–PDK1 fusion protein was incubated with [^{35}S]methionine-labeled JNK or PKCζ, which is known to be phosphorylated by PDK1 (Chou *et al*., 1998; Le Good *et al*., 1998), and binding compared with that of *in vitro* translated [^{35}S]methionine-labeled SGK. Strikingly, no binding was detectable between GST–PDK1 and JNK, while under the same conditions, specific interaction between GST–PDK1 and SGK was readily apparent (Figure 9B, compare lanes 2 and 4). As expected, GST–PDK1 and PKCζ interact *in vitro*, consistent with previous reports of PKCζ being a direct target of PDK1 (Chou *et al*., 1998; Le Good *et al*., 1998). None of the *in vitro* translated products bound GST alone (data not shown).

The specific domains within SGK involved in binding to PDK1 were characterized further by incubating GST–PDK1 with various SGK truncations, and the proteins retained on the beads were analyzed by SDS–PAGE as described previously. The different SGK fragments included full-length wild-type SGK (WT-SGK, 1–431 aa), kinase-dead SGK (K127M-SGK, 1–431 aa), N-terminal deletion mutant of SGK which lacks the first 60 amino acids (aa) (ΔN-SGK, 60–431 aa), C-terminal deletion mutant of SGK that is devoid of 76 aa at the carboxy end (ΔC-SGK, 1–355 aa) and SGK sequences encompassing the catalytic domain (Cat.-SGK, 60–355 aa). Specific binding between GST–PDK1 and SGK was noted with all the fragments tested (Figure 9C, lower panel, compare

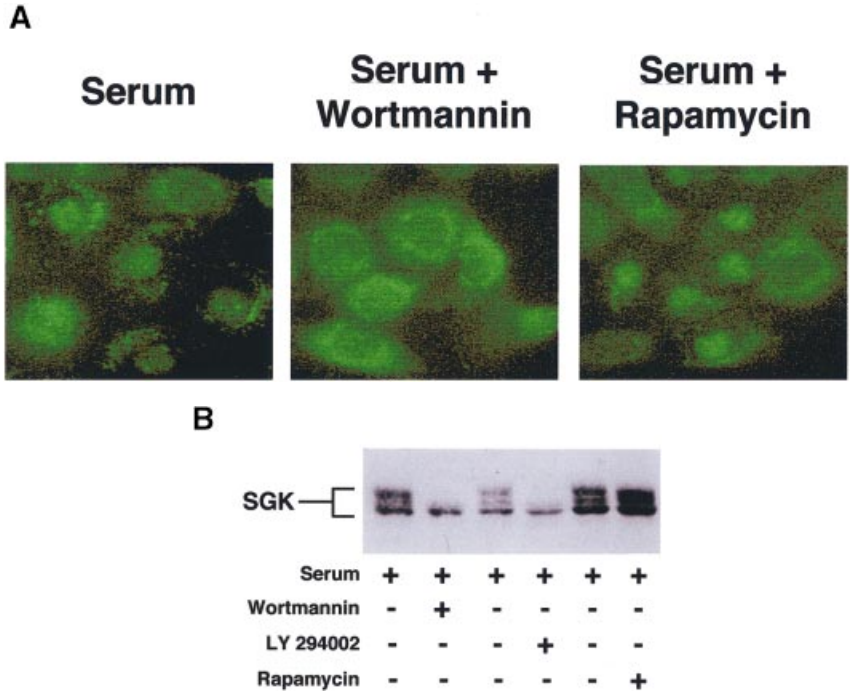

Fig. 10. Intracellular localization and mobility-shift assay of endogenous SGK. Con8.hd6 mammary tumor cells were serum starved for 48 h. During the last 24 h, the cells were pretreated with 100 nM wortmannin or 20 μM rapamycin on cover-slips. After starvation the cells were stimulated for 3 h with 10% FCS/DMEM-F12 in the contained presence of the appropriate inhibitor. (**A**) The cells were stained with affinity-purified rabbit polyclonal anti-SGK antibody, followed by anti-rabbit FITC-conjugated secondary antibody to reveal the intracellular location of the SGK protein. (**B**) After the treatment described in (A), cells were lysed and equal amounts of extracts were electrophoretically fractionated and immunoblotted with the affinity-purified rabbit polyclonal anti-SGK antibody.

lanes 1, 3, 5, 7 and 9 with 2, 4, 6, 8 and 10, respectively), thereby defining the catalytic domain as the region within SGK responsible for mediating interaction with GST–PDK1. The middle panel in Figure 9C indicates 10% of the *in vitro* translated products of the SGK deletions used for the binding assays. Taken together, these data demonstrate direct association between the catalytic domain of SGK with PDK1.

Inhibition of PI 3-kinase signaling disrupts the serum-dependent localization and electrophoretic mobility of endogenous SGK

In serum-stimulated mammary epithelial cells, a hyperphosphorylated form of SGK localizes to the nucleus (Buse *et al.*, 1999). Therefore, to determine whether PI 3-kinase signaling affects the subcellular distribution of endogenous SGK, serum-stimulated Con8.hd6 rat mammary tumor cells were treated either with wortmannin, a selective inhibitor of PI 3-kinase, or as a control with rapamycin, an inhibitor of $p70^{S6K}$ activity. After serum stimulation, SGK localization was examined by indirect immunofluorescence using affinity-purified SGK antibodies. As shown in Figure 10A, exposure to wortmannin prevented the serum-dependent nuclear localization of SGK, which resulted in a cytoplasmically localized form of SGK of this protein kinase. In contrast, treatment with rapamycin did not alter SGK localization compared with the untreated serum-stimulated cells, with ~90% of SGK remaining nuclear under both conditions. Characterization of the electrophoretic mobility of endogenous SGK protein revealed that treatment with either of the two PI 3-kinase inhibitors, wortmannin or LY294002, inhibited the serum-dependent induction of the hyperphosphorylated forms of SGK protein (Figure 10B). Consistent with the localization data, treatment of serum-stimulated cells with rapamycin had no effect in the electrophoretic mobility of SGK. Short-term stimulation of transfected HEK 293 or COS-1 cells revealed that SGK translocated to the nucleus following agonist treatment (data not shown). Taken together, these data establish that endogenous SGK lies downstream of PI 3-kinase signaling, but is not on the same pathway as $p70^{S6K}$.

Discussion

In the present study we have demonstrated that SGK, a novel member of the second-messenger family of serine/threonine protein kinases, is regulated by reversible phosphorylation, apparently mediated by the PI 3-kinase signaling pathway. Furthermore, our findings suggest that PDK1 phosphorylates Thr256 in the activation loop of SGK leading to activation of the kinase both *in vivo* and *in vitro*. Significantly, this work also reveals that SGK is a serine/threonine-specific protein kinase which is activated in response to insulin/IGF-1 stimulation.

We propose that the following events are required for the rapid and efficient activation of SGK. Growth factor binding to their cognate receptors prompts PI 3-kinase activity, thereby generating 3-phosphorylated phosphoinositides leading to the activation of upstream kinase(s), which in turn activates SGK. Furthermore, we propose that PDK1 is one of the immediate upstream regulators of SGK activity as judged by GST-pulldown experiments and phosphorylation data. In this scheme, PP2A would act as a negative regulator of the activating phosphorylation. The data obtained by *in vitro* phosphatase treatment

or *in vivo* calyculin-A treatment are consistent with PP2A being a primary negative regulator of SGK activity (Figures 3 and 4). Therefore, the balance between PDK1 and PP2A activity determines the phosphorylation state and thus activity of SGK. Consistent with this possibility, pervanadate, a broad spectrum phosphatase inhibitor, induces SGK activation and the presence of the hyper-phosphorylated form of SGK. The activation of SGK by pervanadate was sensitive to LY294002, implying that SGK is a cellular target of receptor-activated PI 3-kinase. Furthermore, our results show that serum, insulin or IGF-1 activates SGK through a LY294002- or wortmannin-sensitive mechanism. Activation by these growth factors suggests that SGK is an important part of the insulin and IGF-1 signaling network along with PKB family, $p70^{S6K}$ and some PKCs. Therefore, any physiological response involving insulin signaling should include SGK as part of this signaling cascade.

Identification of the phosphorylation sites, the kinases responsible for modifying them and their association with SGK is of considerable importance in gaining a detailed understanding of the activating mechanism and delineation of the signaling pathway. Based on comparison with the sequences of PKB, PKC, $p70^{S6K}$ and PKA (Figure 1), Thr256 and Ser422 of SGK were predicted to be potential phosphorylation sites involved in the regulation of its kinase activity. Indeed, the mutations of these sites to alanine (T256A, S422A) caused significant decreases in the kinase activity (Figure 7A). The current data indicate that SGK is one of the protein kinases that is apparently robustly activated following insulin or growth factor treatment. Several second-messenger protein kinases are targets of PDK1, and these fall into two classes: inducible or constitutive phosphorylation (for a review see Belham *et al*., 1999). The identity of the kinase responsible for modifying Ser422 remains to be established. Recent data (P.Buse, S.Tran, M.L.L.Leong, J.Park, B.A.Hemmings and G.L.Firestone, in preparation) showed that SGK associates with extracellular signal-related kinase/mitogen-activated protein kinase (ERK/MAPK) following serum stimulation of mammary tumor cells. These results suggest that ERK/MAPK could be responsible for phosphorylating S/TP sites in the N- and C-terminal domains.

None of the endogenous downstream targets of SGK have been identified so far. The identification of the upstream components between PI 3-kinase and SGK and downstream targets will be crucial for understanding this signaling pathway. Moreover, the regulation of SGK activity by PI 3-kinase pathway further implicates SGK in cell survival mechanisms. In this regard we have recently shown that osmotic shock stimulates both SGK expression and enzymatic activity (L.Bell, M.L.L.Leong, J.Park, B.A.Hemmings and G.L.Firestone, unpublished data). Furthermore, recent work has shown that SGK regulates the activity of the epithelial sodium channel in coinjected *Xenopus laevis* oocytes, suggesting a role for SGK in the control of cell volume and sodium homeostasis (Chen *et al*., 1999).

SGK was originally identified as a gene which is induced by serum and glucocorticoids, respectively (Webster *et al.*, 1993a). These effects of serum and glucocorticoids on SGK expression are additive (Webster *et al.*, 1993a,b), suggesting that each modulator acts through a distinct signal transduction pathway. Interestingly, the accumulated data on SGK transcription in different cell type and stimuli (Imaizumi *et al.*, 1994; Maiyar *et al.*, 1996; Alliston *et al.*, 1997; Delmolino and Castellot, 1997; Hollister *et al.*, 1997; Waldegger *et al.*, 1997) indicate that SGK is implicated in two opposite pathways of proliferation and anti-proliferation, respectively. Additionally, recent reports show that the cytoplasmic-nuclear distribution of SGK is differentially regulated depending on the proliferative state of the cells (Buse *et al*., 1999). It is tempting to speculate that steroid or growth factor regulation of protein kinase transcription could be a general mechanism for regulating specific phosphorylation networks, involving cross-talk between a membrane-linked signaling pathway and intracellular steroid hormone activation of gene transcription (Webster *et al*., 1993a). Furthermore, the cytoplasmic-nuclear shuttling of SGK is regulated in synchrony with the cell cycle (Buse *et al*., 1999), suggesting a central role for SGK in proliferation control. We hypothesize that like other early growth-response gene products, SGK plays a role in amplifying the mitogenic signal. However, an intriguing question remains as to why this kinase is also partially transcriptionally regulated (by mRNA induction) rather than being modulated solely post-translationally (by phosphorylation–dephosphorylation) by second messengers and other kinases.

Materials and methods

Construction of expression vectors

HA epitope-tagged constructs (HA-SGK) were prepared by amplifying the rat SGK cDNA in-frame with the initiator methionine (Webster *et al*., 1993a) with primers 5′-CCC GGT ACC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT TCG ACC GTC AAA ACC GAG GCT GCT CGA and 3′-CCC GGT ACC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT. The polymerase chain reaction (PCR) products were subcloned between *Kpn*I and *Xba*I sites of the mammalian expression vector pCMV4. The mutants at Lys127 (kinase-deficient HA-SGK K127M), Thr256 (HA-SGK T256A and HA-SGK T256D), Ser422 (HA-SGK S422A and HA-SGK S422D) or both Thr256 and Ser422 (HA-SGK DD) were created using the Quickchange kit (Stratagene) as described by the manufacturer, with pCMV4 HA-SGK as template. The N- and C-terminal deletions of SGK were constructed in pcDNA3 expression vector using standard PCR-cloning strategies. The Myc-tagged PDK1 and Myc-PDK1-KD, the kinase-deficient mutant of PDK1, were the same as reported previously (Pullen *et al*., 1998). The GST–PDK1 fusion protein was constructed in pGEX-4T1 bacterial expression vector using standard PCR cloning techniques. Membrane-targeted p110 constructs (HA-p110 CAAX and HA-p110 KD CAAX) were the same as reported previously (Didichenko *et al.*, 1996). All constructs were confirmed by automated DNA sequencing. Sequences of the mutagenic oligonucleotides are available upon request.

Cell culture and stimulation

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Life Technologies) at 37°C, in an atmosphere containing 5% CO_2. HEK 293 cells seeded at 10^6/10-cm dish and 0.5×10^6/5-cm dish, respectively, were transfected the following day by a modified calcium phosphate method (Chen and Okayama, 1988), with 1–2 μg/ml plasmid DNA. The transfection mixture was removed after 16 h incubation, and cells were serum starved for 24 h before stimulation for 15 min with 100 nM insulin (Boehringer Mannheim), 50 ng/ml IGF-1 (Life Technologies), 100 nM calyculin-A (Alexis), 100 ng/ml TPA (Life Technologies), 0.2 mM H_2O_2 or 0.1 mM pervanadate prepared with 0.2 mM H_2O_2 (Posner *et al*., 1994). Pretreatment with 50 μM LY294002 (Alexis) was done for 15 min prior to cell stimulation.

The Con8.hd6 mammary epithelial tumor cells were grown to 30% confluency in 6-well tissue culture plates in DMEM-F12 medium with

10% calf serum (Biowhittaker). Cells were serum starved for 48 h and treated with 100 nM wortmannin (Calbiochem), 50 μM LY29004 (Calbiochem) or 50 μM rapamycin (generous gift from B.Webb and G.Steven Martin, University of California at Berkeley, CA) for 24 h. Serum was added in the presence or absence of respective inhibitors for 3 h.

Immunoprecipitation and in vitro kinase assay

Cells were placed on ice and extracted with lysis buffer containing 50 mM Tris–HCl pH 7.5, 1% w/v Nonidet P-40 (NP-40), 120 mM NaCl, 25 mM NaF, 40 mM β-glycerol phosphate, 0.1 mM sodium orthopervanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine and 2 μM microcystin-LR. Lysates were centrifuged for 15 min at 12 000 *g*, and the HA-SGK protein was immunoprecipitated from 400 μg of cell-free extracts with the anti-HA epitope 12CA5 monoclonal antibody coupled to protein A–Sepharose (Pharmacia Biotech.). The immune complexes were washed once with lysis buffer containing 0.5 M NaCl, followed by washing with lysis buffer and finally with kinase assay buffer (50 mM Tris–HCl pH 7.5, 0.1% v/v 2-mercaptoethanol). *In vitro* kinase assays were performed for 60 min at 30°C in 50 μl of reaction volume containing 30 μl of immunoprecipitate in kinase buffer, 1 mM Sgktide (KKRNRRLSVA) as substrate, 10 mM $MgCl_2$, 1 μM PKA inhibitor peptide (Bachem) and 100 μM [γ-^{32}P]ATP (1000–2000 c.p.m./pmol; Amersham).

For the coupled kinase assay, HA-SGK and Myc-PDK1 were independently expressed in HEK 293 cells. Extracts from cells expressing HA-SGK or Myc-PDK1 were immunoprecipitated with the anti-HA (12CA5) or anti-Myc (9E10) monoclonal antibody coupled to protein A–Sepharose or protein G–Sepharose (Pharmacia Biotech.), respectively. HA-SGK was eluted from immune complexes with HA epitope peptide (1 mg/ml) in kinase buffer. The immunoprecipitated Myc-PDK1 was then mixed with eluted HA-SGK in the presence of 10 mM $MgCl_2$ and 100 μM ATP. After incubation for 30 min at 30°C, the protein-G beads were removed by centrifugation, and 1 mM Sgktide and [γ-^{32}P]ATP (1000–2000 c.p.m./pmol) were added. The reaction mixtures were further incubated for 60 min at 30°C to measure the SGK activity. All reactions were stopped by adding 50 μM EDTA and processed as described previously (Andjelkovi *et al.*, 1996). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Protein phosphatase 2A (PP2A) treatment

Immunoprecipitated HA-SGK was incubated with 10 ng of purified recombinant PP2A catalytic subunit (T.Myles and B.A.Hemmings, unpublished data) in 45 μl buffer containing 50 mM Tris–HCl pH 7.5, 1% 2-mercaptoethanol, 1 mM $MnCl_2$, 1 mM benzamidine, 0.5 mM PMSF for 30 min at 30°C. The reactions were stopped by addition of 1 μM okadaic acid. The immune complexes were washed with 50 mM Tris–HCl pH 7.5, 1 mM benzamidine, 0.5 mM PMSF and 1 μM okadaic acid (Alexis), and SGK was assayed as described above.

Immunoblot analysis

HEK 293 cell extracts and immunoprecipitates were resolved by 10% SDS–PAGE and transferred to Immobilon P membranes (Millipore). The filters were blocked for 30 min in 1× phosphate-buffered saline (PBS) containing 5% skimmed milk, 0.5% Triton X-100 and 0.5% Tween-20, followed by a 2 h incubation with the anti-HA epitope 12CA5 monoclonal antibody diluted 1000-fold in the same blocking solution. The secondary antibody was alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) diluted 2500-fold in the blocking buffer. The detection and quantitation of SGK expression was carried out by using the AP color development reagents from Bio-Rad.

Soluble whole-cell extracts from Con8.hd6 mammary epithelial cells were grown under the conditions indicated and resolved by SDS–PAGE. Proteins were transferred to Nytran membranes (Schleicher & Schuell). The membrane was probed with a 1:2500 dilution of polyclonal anti-SGK antibody (Webster *et al.*, 1993a) in 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20 with 1% non-fat dry milk. The secondary antibody was a goat anti-rabbit IgG HRP-conjugated antibody (Bio-Rad). The Western blot was developed by using the Renaissance developing kit (NEN, Boston, MA) and exposure to film.

In vitro transcription, and translation of SGK

In vitro transcription and translation of full-length wild-type SGK (WT-SGK, 1–431 aa), kinase-dead SGK (K127M-SGK, 1–431 aa), N- and C-terminal deleted SGK (ΔN-SGK, 60–431 aa; ΔC-SGK, 1–355 aa) and catalytic domain only SGK (Cat.-SGK, 60–355 aa) was performed using the TNT-coupled rabbit reticulocyte kit (Promega) according to the manufacturer's instructions. The expression plasmids encoding JNK protein and PKCζ were kindly provided by J.S.Gutkind (Molecular signaling unit, National Institute of Dental Research NIH, Bethesda, MD) and have been described previously (Crespo *et al.*, 1995).

Expression of GST fusion protein in bacterial system

The GST–PDK1 fusion protein was isolated from AB1899 cells transformed with GST–PDK1 expression plasmid. Briefly, bacteria were initially grown at 37°C for 2 h (OD_{600} = 0.5–0.7) and subsequently induced with 0.5 mM isopropyl-thio-β-D-galactopyranoside (IPTG) for 16 h at 30°C. Cells were lysed using the French Press (three times) in lysis buffer (PBS containing 0.05% Tween-20, 2 mM EDTA, 1 mM DTT and 0.1% 2-mercaptoethanol). The GST–PDK1 fusion protein was purified on glutathione–agarose beads (Pharmacia) as described previously (Chakraborty *et al.*, 1991).

Binding of GST–PDK-1 and in vitro translated SGK

In order to examine the interaction between PDK1 and SGK *in vitro*, 25 μg GST–PDK1 immobilized on glutathione–Sepharose beads were incubated with 5 μl of [^{35}S]methionine-labeled SGK *in vitro* translation product in 180 μl of binding buffer (20 mM HEPES–KOH pH 7.9, 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.2% NP-40, 1.5 mM PMSF, and 3 μl of normal goat serum/180 μl binding buffer). The slurry was incubated overnight at 4°C on a nutator, following which the beads were washed five times in wash buffer (200 mM NaCl, 0.2% Tween 20, 10 mM Tris pH 7.5 and 0.5% non-fat dry milk). After removing the supernatant in the final wash, samples were resuspended in 25 μl of 2× SDS sample buffer, boiled for 5 min and the proteins retained on the beads resolved by SDS–PAGE. Binding was compared with that of 10% of the *in vitro* translated products added to the binding reactions. Gels were dried at 60°C and autoradiography carried out at –70°C.

Indirect immunofluorescence microscopy

Con8.dh6 mammary tumor cells were cultured on 8-well Lab-Tek Permanox slides (Nalgene) and grown to 30% confluency before the indicated combinations of serum and/or wortmannin or rapamycin were added for 24 h. Cell confluency prior to fixation did not exceed 60%. Cells were washed with PBS, fixed for 15 min in 3.7% formaldehyde/0.1% glutaraldehyde, rinsed with PBS and permeabilized with 50% methanol/50% acetone for 1 min. Following a rinse in PBS, the cells were preabsorbed for 5 min in PBS containing 4% normal goat serum (Jackson Immuno Research Laboratories). The cells were incubated in a 1:300 dilution of affinity-purified rabbit polyclonal anti-SGK antibody for 1–2 h at 25°C. After five washes with PBS, cells were treated for 5 min with PBS containing 4% normal goat serum. The cells were incubated with a 1:300 dilution of anti-rabbit FITC-conjugated secondary antibody (Cappel Research Products) in PBS and then incubated for 30 min at 25°C. Cells were washed 5 times with PBS and mounted with 50% glycerol, 50 mM Tris–HCl pH 8.0 containing 4 mg/ml *N*-propyl gallate, and examined under a Nikon Optiphot fluorescence microscope. Images were captured using Adobe Photoshop 3.0.5 (Adobe Systems) and a Sony DKC-5000 digital camera. Non-specific fluorescence was determined by incubation with the secondary antibody alone and shown to be negligible.

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