Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2

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The PtdIns(3,4,5)P_3-dependent activation of protein kinase B (PKB) by 3-phosphoinositide-dependent protein kinases-1 and -2 (PDK1 and PDK2 respectively) is a key event in mediating the effects of signals that activate PtdIns 3-kinase. The catalytic domain of serum- and glucocorticoid-regulated protein kinase (SGK) is 54% identical with that of PKB and, although lacking the PtdIns(3,4,5)P_3-binding pleckstrin-homology domain, SGK retains the residues that are phosphorylated by PDK1 and PDK2, which are Thr^{256} and Ser^{422} in SGK. Here we show that PDK1 activates SGK *in vitro* by phosphorylating Thr^{256}. We also show that, in response to insulin-like growth factor-1 (IGF-1) or hydrogen peroxide, transfected SGK is activated in 293 cells via a PtdIns 3-kinase-dependent pathway that involves the phosphorylation of Thr^{256} and Ser^{422}. The activation of SGK by PDK1 *in vitro* is unaffected by PtdIns(3,4,5)P_3, abolished by the mutation of Ser^{422} to Ala, and greatly potentiated by mutation of Ser^{422} to Asp (although this mutation does not activate SGK itself). Consistent with these findings, the Ser^{422}Asp mutant of SGK is activated by phosphorylation (probably at Thr^{256}) in unstimulated 293 cells, and activation is unaffected by inhibitors of PtdIns 3-kinase. Our results are consistent with a model in which activation of SGK by IGF-1 or hydrogen peroxide is initiated by a PtdIns(3,4,5)P_3-dependent activation of PDK2, which phosphorylates Ser^{422}. This is followed by the PtdIns(3,4,5)P_3-independent phosphorylation at Thr^{256} that activates SGK, and is catalysed by PDK1. Like PKB, SGK preferentially phosphorylates serine and threonine residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr motifs, and SGK and PKB inactivate glycogen synthase kinase-3 similarly *in vitro* and in co-transfection experiments. These findings raise the possibility that some physiological roles ascribed to PKB on the basis of the overexpression of constitutively active PKB mutants might be mediated by SGK.

Key words: insulin, protein kinase B.

INTRODUCTION

Protein kinase B (PKB) (also called c-Akt or 'related to A and C' kinase, otherwise known as RAC kinase) is believed to play a key role in mediating many of the metabolic actions of insulin, as well as the anti-apoptotic effects of survival factors such as insulin-like growth factor-1 (IGF-1) (reviewed in [1,2]). PKB is activated within a few minutes in response to insulin or survival factors via a PtdIns 3-kinase-dependent mechanism [3–5]. Activation results from the phosphorylation of Thr^{308} and Ser^{473}, and the phosphorylation of both residues is prevented by inhibitors of PtdIns 3-kinase [6]. Thr^{308}, which is located in the 'activation loop' between subdomains VII and VIII of the catalytic domain, is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) [7–9], whereas Ser^{473} lies close to the C-terminus and is phosphorylated by a distinct enzyme termed PDK2 that has yet to be characterized.

The activation of PKB by PDK1 *in vitro* only takes place in the presence of lipid vesicles containing PtdIns(3,4,5)P_3, the product of the PtdIns 3-kinase reaction, and results from the interaction of this 'second messenger' with the pleckstrin homology (PH) domains of both PKB and PDK1 [9,10]. Thus removal of the PH domain of PDK1 (PDK1-ΔPH), or point mutations that prevent its interaction with PtdIns(3,4,5)P_3, greatly reduce the rate of activation of PKB [11]. However, the slow activation of PKB catalysed by PDK1-ΔPH still has an absolute requirement for PtdIns(3,4,5)P_3. This requirement is removed by deletion of the PH domain of PKB [9–11].

PtdIns(3,4,5)P_3 is associated with the inner leaflet of the plasma membrane. The insulin- or IGF-1-induced increase in PtdIns(3,4,5)P_3 is therefore accompanied by the recruitment of PKB from the cytosol to the plasma membrane, where it becomes activated [12,13]. Membrane recruitment appears to facilitate activation by PDK1 and PDK2 [12], and a significant proportion of the PDK1 is associated with the plasma membrane of unstimulated cells [11]. PDK1 binds to PtdIns(3,4,5)P_3 much more avidly than does PKB [10,11], and an interaction of PDK1 with the low levels of PtdIns(3,4,5)P_3 present under basal conditions might explain, at least in part, its constitutive association with the plasma membrane. However, we have been unable to demonstrate any translocation of PDK1 from the cytosol to the plasma membrane in response to IGF-1 or platelet-derived growth factor [11], in contrast with another recent report [14].

The amino acid sequence immediately C-terminal to Thr^{308} of PKB is similar to that found in other protein kinases that play

Abbreviations used: EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; GSK3, glycogen synthase kinase-3; GST, glutathione S-transferase; GST–ΔN-SGK[S422D], an N-terminally truncated SGK in which Ser^{422} has been mutated to Asp; MALDI–TOF, matrix-assisted laser desorption ionization–time-of-flight; PDGF, platelet-derived growth factor; PDK, 3-phosphoinositide-dependent protein kinase; PH, pleckstrin homology; PKB, protein kinase B; PKC, protein kinase C; PKI, specific peptide inhibitor of cAMP-dependent protein kinase; PP2A, protein phosphatase 2A; PtdIns, phosphatidylinositol; SGK, serum- and glucocorticoid-regulated protein kinase.

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                256                                  422
SGK       TTSTFCGTPEYLAPE.......FLGFSYAPP

                308                                  473
PKBα      TMKTFCGTPEYLAPE.......FPQFSYSAS

                229                                  389
p70 S6K   VTHTFCGTIEYMAPE.......FLGFTYVAP

                507                                  664
PKCδ      RASTFCGTPDYIAPE.......FAGFSFVNP
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Figure 1 Amino acid sequences surrounding the activating phosphorylation sites on PKB, p70 S6 kinase and PKCδ, and their similarity to the corresponding regions of SGK

Identities are shown in bold-face type and the phosphorylated residues in PKB and p70 S6 kinase (S6K) are underlined. The phosphorylation sites are separated by 160–165 residues in each enzyme.

important roles in signal transduction, such as p70 S6 kinase and protein kinase C (PKC) (Figure 1). Indeed, PDK1 activates p70 S6 kinase [15,16], PKCδ and PKCζ [17,18] *in vitro* and in co-transfection experiments, by phosphorylating the residue equivalent to Thr^{308} of PKB. The activation of p70 S6 kinase by PDK1 *in vitro* is not affected by PtdIns(3,4,5)P_3.

Like PKB, p70 S6 kinase and PKC contain the consensus sequence for phosphorylation by PDK2 (Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr), which is always located 160–165 residues C-terminal to the PDK1 phosphorylation site (Figure 1). These observations suggest that PDK1 and PDK2 might participate together in the activation of a number of protein kinases.

Serum- and glucocorticoid-regulated protein kinase (SGK) was originally identified in a differential screen aimed at finding glucocorticoid-inducible transcripts [19]. The levels of SGK mRNA and protein are both elevated 5–10-fold, with a half-time of 30 min in Rat2 fibroblasts, following cell stimulation with serum or glucocorticoids [20]. SGK is also induced in response to other stimuli, such as follicle-stimulating hormone [21], increased extracellular osmolarity [22], injury of the brain [23,24] and by transfection of mammary epithelial cells with p53 [25]. However, the glucocorticoid-stimulated induction of SGK is suppressed by wild-type p53 in Rat2 fibroblasts, but not by mutant p53 [26].

The structure of SGK is most similar to PKB, although it lacks a PH domain. The catalytic domain of SGK is 54% identical with PKB, 50% identical with p70 S6 kinase and 48% identical with PKC. Interestingly, SGK possesses a threonine residue (Thr^{256}) at the position equivalent to Thr^{308} of PKB and a serine residue (Ser^{422}) equivalent to Ser^{473}, and the sequences surrounding these residues suggest that they might be phosphorylated by PDK1 and PDK2 (Figure 1). In the present study we demonstrate that, like PKB, SGK is activated by IGF-1, or by oxidative stress in 293 cells, via a PtdIns 3-kinase-dependent pathway(s). Activation requires the phosphorylation of Thr^{256} and Ser^{422}, and Thr^{256} is phosphorylated by PDK1 *in vitro*.

MATERIALS AND METHODS

Materials

Human PDK1 [9] and human PKBα [7] were expressed as glutathione-S-transferase (GST) fusion proteins in 293 cells and purified on glutathione–Sepharose. A pCMV5 DNA construct encoding wild-type glycogen synthase kinase-3 (GSK3), to which the epitope EEMPME had been added at the N-terminus (WT-EE–GSK3), was prepared as described in [27], and a pCMV5 DNA construct encoding a Myc-tagged mutant of GSK3β in which Ser^9, the residue targeted by PKB, had been changed to Ala (A9-Myc–GSK3) was generously provided by Dr Michel Goedert (MRC Laboratory for Molecular Biology, Cambridge, U.K.). V8 protease (protease Glu-C) and complete protease-inhibitor cocktail were purchased from Boehringer Mannheim (Mannheim, Germany). Residues 5–24 of the specific protein inhibitor of cAMP-dependent protein kinase (PKI) and all other peptides were synthesized at the Department of MRC Protein Phosphorylation Unit, University of Dundee, by Mr. F. B. Caudwell. Sources of other materials are given in [6].

Buffer solutions

Buffer A comprised 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose and 0.1% (v/v) 2-mercaptoethanol. Lysis buffer was Buffer A containing 1 μM microcystin-LR, plus complete protease-inhibitor cocktail (one tablet/50 ml). Buffer B comprised 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol.

Plasmids expressing SGK and site-directed mutagenesis

A full-length cDNA encoding human SGK from an infant brain library was obtained from I.M.A.G.E. Consortium (clone ID 42669) and modified by PCR amplification to introduce a *Bam*HI site at the 5′-end (in-frame with the GST sequence of the pEBG2T vector), and a *Kpn*I site at the 3′-end using oligonucleotides 5′-ACA CGG ATC CGC CAC CAT GTA TCC ATA TGA TGT TCC AGA TTA TGC TAC GGT GAA AAC TGA GGC TGC TAA GGG C-3′ and 5′-ACA CGG TAC CGT CGA CTC AGA GGA AAG AGT CCG TGG GAG G-3′. The PCR product was digested with *Bam*HI and *Kpn*I and inserted into the cloning site of pEBG-2T. A truncated form of SGK (ΔN-SGK) lacking the N-terminal 60 residues was obtained by removing the fragment between the *Bam*HI site at the 5′-end of the cDNA and a *Bgl*II site located 175 bp from the initiation codon, followed by re-ligation after filling the gap with a Klenow fragment. All of the point mutations in the SGK gene were introduced by *in vitro* mutagenesis using PCR.

Expression of SGK in 293 cells

Cells were transfected with 10 μg (10-cm dish) or 3 μg (6-cm dish) of the pEBG-SGK constructs using a modified calcium-phosphate gel method [6]. At 24 h after transfection, the cells were deprived of serum for 16 h, then incubated for 10 min with or without 100 nM wortmannin, or for 1 h with or without other inhibitors, followed by stimulation with 50 ng/ml IGF-1 or 2 mM hydrogen peroxide. The cells were lysed in 1 ml of ice-cold lysis buffer, centrifuged for 5 min at 13000 ***g*** and GST–SGK was purified on glutathione–Sepharose, as described for PKB [6]. The glutathione–Sepharose eluate was stored in aliquots at −80 °C.

Phosphorylation and assay of SGK

Phosphorylation was carried out at 30 °C in 50 μl incubations containing 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 2.5 μM PKI, 1 μM microcystin-LR, 10 mM magnesium chloride and 100 μM ATP. Where indicated, lipid vesicles containing phosphatidylcholine (100 μM), phosphatidylserine (100 μM) and PtdIns(3,4,5)P_3 (1 μM) were also included. SGK activity was assayed exactly as described for PKB [6] using the peptide GRPRTSSFAEG ('Crosstide') at 30 μM as

substrate. A unit of activity was defined as that amount which catalysed the phosphorylation of 1 nmol of 'Crosstide' in 1 min.

Mapping the site on SGK phosphorylated by PDK1

The N-terminally truncated SGK, in which Ser^{422} had been mutated to Asp (GST–ΔN-SGK[S422D]) (26 μg), was incubated for 20 min at 30 °C with protein phosphatase 2A (PP2A; 30 m-units/ml), where one unit of activity is that amount which catalyses the dephosphorylation of 1 nmol of phosphorylase *a* in 1 min. After addition of microcystin-LR to 1 μM to inactivate PP2A, the SGK was phosphorylated by incubation for 30 min at 30 °C with GST–PDK1 (2 μg) and [γ-^{32}P]ATP (5000 c.p.m./pmol). The reaction was stopped after 30 min by adding SDS and 2-mercaptoethanol to final concentrations of 1 % (w/v) and 1 % (v/v) respectively, followed by heating at 95 °C for 5 min. The sample was then incubated for 1 h at 30 °C with 2 % (v/v) 4-vinylpyridine to alkylate cysteine residues, electrophoresed on an SDS/10 % polyacrylamide gel, and the ^{32}P-labelled SGK was eluted from the gel and precipitated by the addition of trichloroacetic acid to 20 % (w/v). The precipitated protein was washed six times with 0.2 ml of water, resuspended in 0.3 ml of 50 mM NH_4HCO_3, pH 7.8, digested for 18 h with V8 protease (1 μg) and then chromatographed on a Vydac C18 column, as described in the Results section. Peptide fractions were analysed on a PerSeptive Biosystems (Framingham, MA, U.S.A.) Elite STR matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometer in the linear and reflector mode, using 10 mg/ml α-cyanocinnamic acid as the matrix. Phosphoamino acid analysis was carried out as described previously [28].

Development of a phospho-specific antibody that recognizes SGK phosphorylated at Ser^{422}

The phosphopeptide VKEAAEAFLGFS(*P*)YAPDTD, corresponding to residues 411–428 of SGK, was coupled with BSA and keyhole limpet haemocyanin, and injected into a sheep at the Scottish Antibody Production Unit (Carluke, Ayrshire, U.K.), and a second injection was made 6 weeks later. After a further 2 weeks, the serum was withdrawn, affinity-purified by sequential chromatographies on phosphopeptide-CH-Sepharose and dephosphopeptide-CH-Sepharose, and then incubated with 0.3 mM dephosphopeptide to ensure that all traces of antibody recognizing the dephosphoprotein were eliminated. A further antibody that recognizes phosphorylated and dephosphorylated SGK was made in a similar manner against the synthetic peptide LGFS-YAPPTDSFL corresponding to the C-terminal 13 residues of SGK. Horseradish-peroxidase-conjugated anti-(sheep IgG) were purchased from Pierce and Warriner Ltd. (Chester, U.K.), and immunoblotting was performed as described previously [29].

RESULTS

Expression of SGK in 293 cells

Initial attempts to express SGK with a haemaglutinin tag at its N-terminus were unsuccessful because the expressed protein was insoluble. Our studies were therefore performed using GST-fusion proteins that were soluble. Full-length GST–SGK (Figure 2A, lane 2) was expressed in 293 cells at a much lower level than GST–PKBα (Figure 2A, lane 3). This low level of expression appears to be due to the N-terminal 60 residues of SGK, because their removal increased the level of expression 50–250-fold (Figure 2B, lanes 4 and 5). Full-length SGK that was 20 % pure (10–50 μg) and 0.5 mg of 80 % pure ΔN-SKG(61–431) were usually obtained from ten 10-cm diameter dishes of cells.

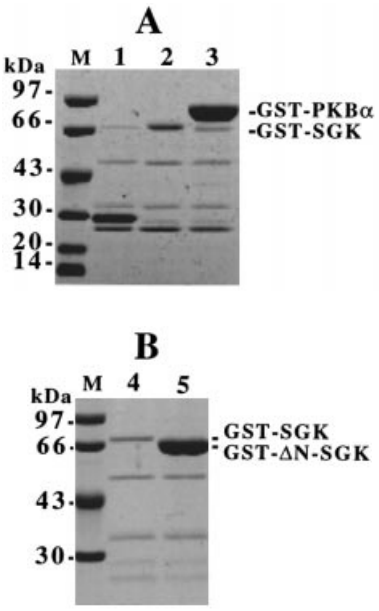

Figure 2 Expression and purification of SGK and PKB fusion proteins in 293 cells

GST-fusion proteins expressed in 293 cells were purified on glutathione–Sepharose from 200 μg of cell lysate protein, subjected to SDS/PAGE on 5–20 % (**A**) or 10 % (**B**) polyacrylamide gels, and stained with Coomassie Blue. Cells were transfected with the following constructs: lane 1, pEBG2T expressing GST; lanes 2 and 4, pEBG-SGK expressing full-length SGK; lane 3, pEBG-PKBα expressing full-length PKBα; lane 5, pEBG-ΔN-SGK(61–431) expressing the N-terminally truncated form of SGK. The marker proteins (lanes M) and their molecular masses are also shown.

Phosphorylation and activation of wild-type SGK by PDK1 *in vitro*

The activity of wild-type GST–SGK purified from 293 cells decreased by 70 % upon incubation with PP2A (Figure 3), a

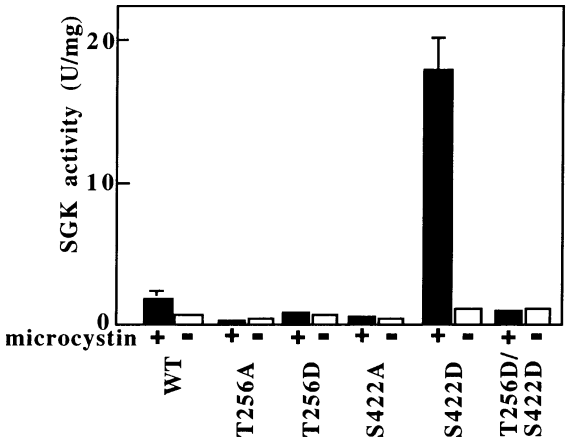

Figure 3 Activity of GST–SGK fusion proteins purified from unstimulated 293 cells

Wild-type SGK and the mutants indicated were expressed in 293 cells and purified on glutathione–Sepharose. Each fusion protein (1 μg) was incubated for 20 min at 30 °C with PP2A (30 m-units/ml) in 50 μl of 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1 % (v/v) 2-mercaptoethanol. Where indicated (+), microcystin-LR (1 μM) was included before the addition of PP2A. In the other incubations (−), microcystin-LR was only added after treatment with the phosphatase. SGK activity was then measured using 'Crosstide' as substrate.

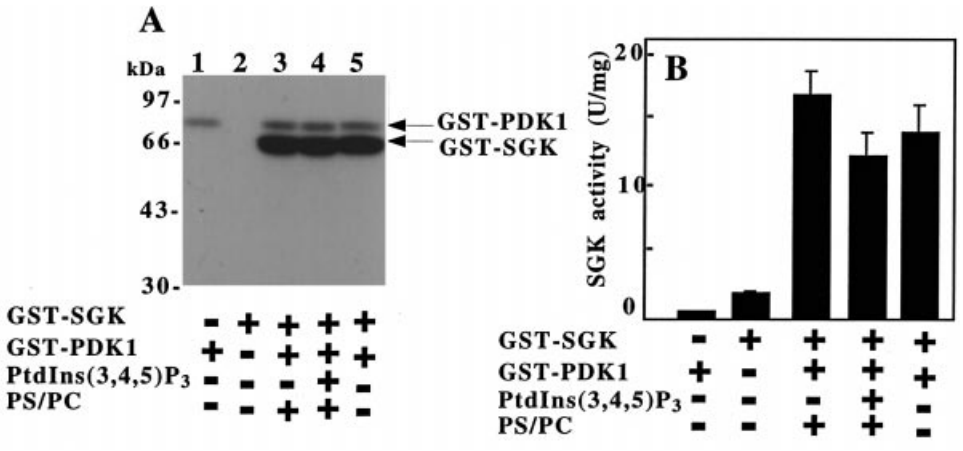

Figure 4 Phosphorylation and activation of SGK by PDK1 *in vitro*

(**A**) GST–SGK (0.3 μg) was phosphorylated using GST–PDK1 (0.1 μg) and [γ-^{32}P] ATP (500 c.p.m./pmol) as described in the Materials and methods section. Where indicated (+), 1μM PtdIns(3,4,5)P_3 (D-enantiomer) in lipid vesicles containing phosphatidylserine (100 μM) and phosphatidylcholine (100 μM) (PS/PC) was also included. The reactions were stopped by the addition of SDS to 2% and, after heating for 5 min at 95 °C, the samples were subjected to electrophoresis on SDS/10% polyacrylamide gels, stained with Coomasssie Blue and then autoradiographed. The positions of the molecular-mass markers are also indicated. (**B**) Same as (**A**), except that unlabelled ATP was used instead of [γ-^{32}P]ATP. After incubation for 10 min at 30 °C, 10-μl aliquots were assayed for SGK activity using 'Crosstide' as substrate. The results are shown as bars ± S.D. for three separate experiments.

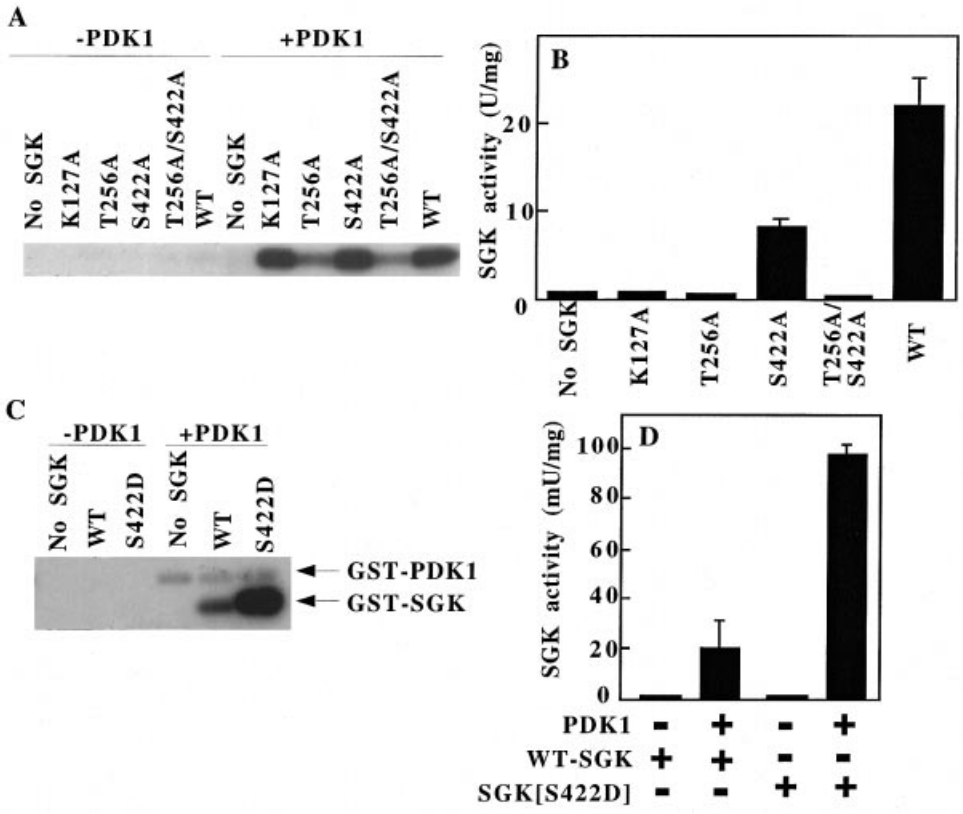

Figure 5 Effect of mutations on the phosphorylation of SGK by PDK1 *in vitro*

(**A**) GST-fusion proteins of wild-type (WT) or mutant SGK (0.3 μg) were incubated with PP2A, as described in the legend to Figure 3 and, after the addition of microcystin-LR (1 μM) to inactivate the PP2A, the SGK proteins were incubated with [γ-^{32}P]ATP (500 c.p.m./pmol) with (+) and without (−) PDK1 (0.1 μg), subjected to SDS/PAGE, stained with Coomasssie Blue and then autoradiographed. The Lys^{127}Ala mutation creates a catalytically inactive mutant. (**B**) Same as (**A**), except that unlabelled ATP was used instead of [γ-^{32}P]ATP (500 c.p.m./pmol). After incubation for 10 min at 30 °C, 10-μl aliquots were assayed for SGK activity using 'Crosstide' as the substrate. The results are shown ± S.D. for three separate experiments. (**C**) GST-fusion proteins of wild-type SGK or the Ser^{422}Asp mutant (1 μg) were incubated with PP2A, and phosphorylated with PDK1 (0.2 μg) and [γ-^{32}P]ATP as in (**A**), then subjected to SDS/PAGE followed by autoradiography. (**D**) Same as (**C**), except that unlabelled ATP was used instead of [γ-^{32}P]ATP. After incubation for 10 min at 30 °C, aliquots of the reactions were assayed for SGK activity. The results are shown ± S.D. for three separate experiments.

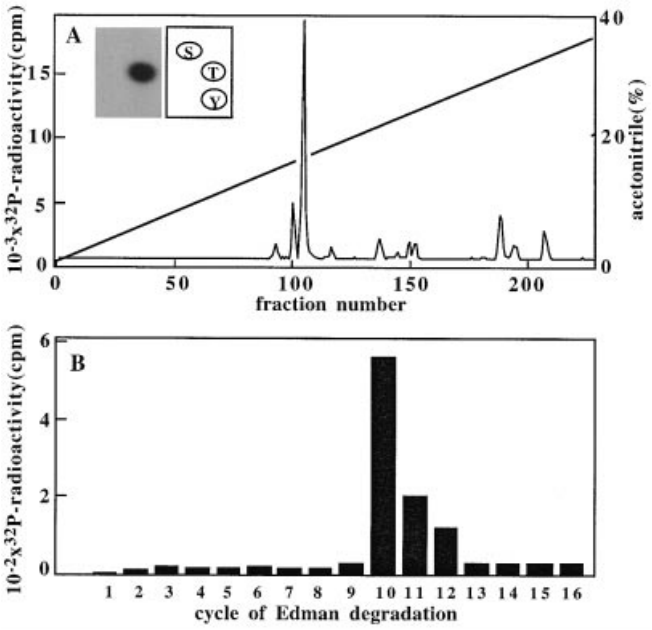

Figure 6 Mapping the PDK1 phosphorylation site on SGK

(**A**) GST–ΔN-SGK[S422D] (26 μg) was phosphorylated with PDK1 and digested with V8 protease, as described in the Materials and methods section. The digest was applied to a Vydac C_{18} column equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed with a linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.8 ml/min, and fractions of 0.4 ml were collected. ^{32}P Radioactivity is shown by the plot, and the acetonitrile gradient by the diagonal line. The inset shows phosphoamino acid analysis of the major ^{32}P-labelled peptide (S, serine; T, threonine; Y, tyrosine). (**B**) An aliquot of the major phosphopeptide in (**A**) was analysed by solid-phase sequencing on an Applied Biosystems 470A sequencer [32] and ^{32}P radioactivity released after each cycle of Edman degradation was measured.

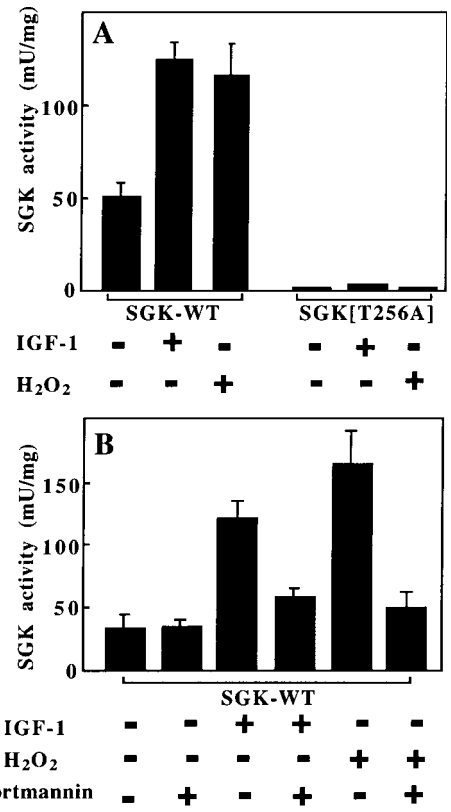

Figure 7 Activation of SGK in 293 cells

(**A**) Cells were transiently transfected with DNA constructs expressing wild-type GST–SGK (SGK-WT) or GST–SGK[T256A], then stimulated for 10 min with 50 ng/ml IGF-1 or for 25 min with 2 mM H_2O_2, followed by lysis in ice-cold lysis buffer. Glutathione–Sepharose (5 μl) was added to 50 μg of cell-lysate protein and, after end-over-end rotation for 30 min at 4 °C, the suspension was centrifuged for 1 min at 13000 ***g***. The supernatant was discarded and the beads were washed four times with 1 ml of Buffer A containing 0.5 M NaCl, then three times with Buffer B, as described in the Materials and methods section. A solution containing 60 mM Tris/HCl, pH 7.5, 0.12 mM EGTA, 0.12% (v/v) 2-mercaptoethanol, 3.0 μM PKI, 24 mM glutathione, 1.2 μM microcystin-LR, 12 mM $MgCl_2$ and 36 μM 'Crosstide' (40 μl) was added to the beads. After leaving on ice for 10 min, the beads were assayed for SGK activity at 30 °C by the addition of 5 μl of 1 mM [γ-^{32}P]ATP (500 c.p.m./pmol). The expression of wild-type and mutant SGKs in 293 cells were similar, as judged by SDS/PAGE followed by staining with Coomassie Blue (results not shown). The results are presented ± S.D. for three separate experiments. (**B**) The experiment was performed with wild-type GST–SGK (SGK-WT) as in (**A**), except that the cells were pretreated for 10 min with 100 nM wortmannin before stimulation with IGF-1 or H_2O_2.

serine/threonine-specific phosphatase, and inactivation was prevented by microcystin, a specific PP2A inhibitor [30]. Thus the basal activity of SGK results from its phosphorylation at one or more serine/threonine residues.

GST–SGK could be phosphorylated by PDK1 (Figure 4A), and phosphorylation was accompanied by a 10-fold increase in the basal activity (Figure 4B). Almost the same level of activity was attained if GST–SGK was first inactivated by treatment with PP2A and then phosphorylated with PDK1 (results not shown). However, PDK1 phosphorylated GST–SGK much more slowly than PKBα, and the maximal stoichiometry of phosphorylation that could be attained was 0.05 mol/mol, compared with 0.5 mol/mol for PKBα. Moreover, unlike the activation of PKBα, the phosphorylation (Figure 4A) and activation (Figure 4B) of SGK was unaffected by PtdIns(3,4,5)P_3 in the presence or absence of lipid vesicles containing phosphatidylserine and phosphatidylcholine.

Effect of mutation of Ser^{422} and Thr^{256} on the activity of SGK

PKBα can be partially activated (5-fold) by either the mutation of Thr^{308} to Asp or the mutation of Ser^{473} to Asp, and almost fully activated (20-fold) when both mutations are combined. It was therefore of interest to study the effect of the equivalent mutations on the activity of SGK.

GST–SGK[T256D], GST–SGK[T256E] or GST–SGK[T256A] were all much less active than wild-type SGK, the activities being similar to wild-type SGK that had been treated with PP2A (Figure 3). In contrast, GST–SGK[S422D] purified from 293 cells had a specific activity that was more than 10-fold higher than that of the wild-type enzyme (Figure 3). However, the activity of GST–SGK[S422D] did not result from the mutation of Ser^{422} to Asp itself, but from an increased phosphorylation, because it could be decreased to the same level as GST–SGK[T256D] or GST–SGK[T256A] by incubation with PP2A (Figure 3).

GST–SGK [S422A] purified from 293 cells also had very low activity, similar to GST–SGK [T256A] or the wild-type enzyme after treatment with PP2A (Figure 3).

Effect of mutation of Ser^{422} or Thr^{256} on the phosphorylation of SGK by PDK1 *in vitro*

The mutation of Ser^{422} to Ala did not affect the extent of phosphorylation of SGK by PDK1 (Figure 5A), but decreased the extent of activation by 60% (Figure 5B). In contrast, the

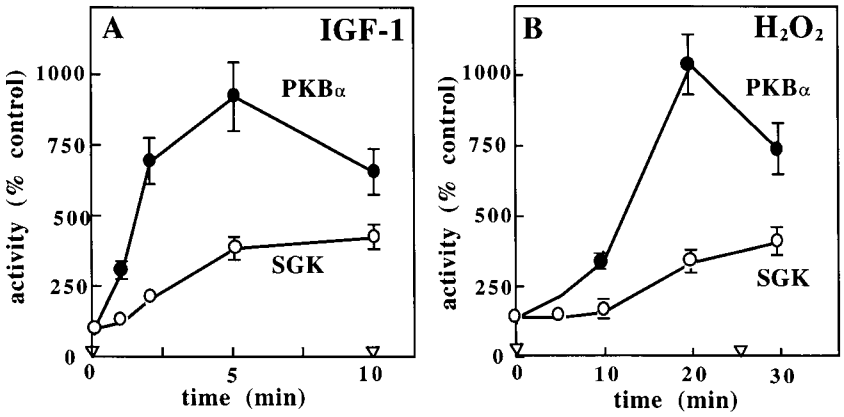

Figure 8 Time course of activation of SGK in 293 cells

293 cells were transiently transfected with pEBG-SGK (○) or pEBG-PKBα (●) and then stimulated with 50 ng/ml IGF-1 (**A**) or 2 mM H_2O_2 (**B**). At the times indicated, the cells were lysed and the GST–SGK or GST–PKBα fusion proteins were purified on glutathione–Sepharose (as in Figure 7) and assayed. GST–SGK activity (▽) after incubation with PP2A (30 m-units/ml) as described in the legend to Figure 3 is also shown in both (**A**) and (**B**). The results are presented as means ± S.D. for a single experiment in which three separate dishes of cells were used at each time point. Similar results were obtained in another independent experiment.

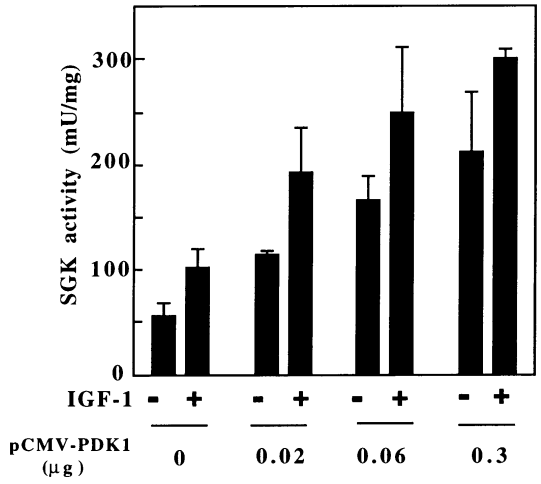

Figure 9 Cotransfection of SGK and PDK1 in 293 cells

pEBG-SGK (3 μg of DNA per 6-cm dish) was transfected into 293 cells as described in [6] in the absence or presence of the indicated amounts of pCMV-Myc-PDK1. After transfection (24 h), the cells were stimulated for 10 min with IGF-1 (50 ng/ml) or left unstimulated and, after cell lysis, GST–SGK was affinity-purified on glutathione–Sepharose, as described in the legend to Figure 7, and assayed. The amount of SGK expressed was similar in each transfection, as assessed by immunoblotting (results not shown).

mutation of Thr^{256} to Ala (or mutation of both Thr^{256} and Ser^{422} to Ala) decreased phosphorylation by 80–90% (Figure 5A) and abolished activation by PDK1 (Figure 5B).

The mutation of Ser^{422} to Asp stimulated the extent of phosphorylation (Figure 5C) and activation (Figure 5D) by PDK1 at least 6-fold. In contrast, the GST–SGK [T256A/S422D] double mutant was not activated at all by PDK1, and phosphorylation by PDK1 was decreased by 80–90% (results not shown).

We also mutated Lys^{127} in the ATP-binding site to Ala to create a 'kinase-dead' protein. This mutant was phosphorylated at the same rate, and to the same extent, as wild-type SGK (Figure 5A) and, as expected, could not be activated by PDK1 (Figure 5B).

Identification of Thr^{256} as the residue on SGK phosphorylated by PDK1 *in vitro*

The results described above suggested that the activation of SGK by PDK1 was likely to result from the phosphorylation of Thr^{256}. In order to investigate whether this was so, we used a form of SGK that was truncated at its N-terminus (GST–ΔN-SGK[61–431]) because this mutant was expressed at far higher levels than the wild-type enzyme, and Ser^{422} was also mutated to Asp to facilitate phosphorylation by PDK1. Like GST–SGK[S422D] (Figure 3), GST–ΔN-SGK(61-431)[S422D] purified from 293 cells had a high activity, which was virtually abolished by treatment with PP2A (results not shown).

PP2A-treated GST–ΔN-SGK[S422D] was phosphorylated by PDK1 to a stoichiometry of 0.6 mol/mol of protein, and the ^{32}P-labelled enzyme was digested with V8 protease and chromatographed on a C_{18} column. One major peak and several minor peaks of ^{32}P radioactivity were observed, as shown in Figure 6(A). Analysis of the major peak by MALDI–TOF-MS showed that its molecular mass (1923.15 Da) was identical with that expected for the peptide comprising residues 247–262 of SGK (NIEHNSTTSTFCGTPE) containing one phosphorylated residue. Residue 247 is preceded by glutamic acid, as expected from the specificity of V8 protease. Phosphoamino acid analysis indicated that this peptide contained only phosphothreonine (inset to Figure 6A). When this phosphopeptide was subjected to solid-phase sequencing, ^{32}P radioactivity was released after the tenth cycle of Edman degradation, corresponding to Thr^{256} (Figure 6B). These results, together with those obtained using the Thr^{256}Ala mutant (Figure 5A) indicate that SGK is phosphorylated by PDK1 at Thr^{256}, the residue equivalent to Thr^{308} of PKBα.

Activation of SGK in 293 cells by extracellular signals

PKB is activated in 293 cells in response to agonists that activate PtdIns 3-kinase, such as insulin or IGF-1 or certain adverse stimuli (e.g. heat shock and oxidative stress). Since the activation of PKB results from the phosphorylation of Thr^{308} and Ser^{473},

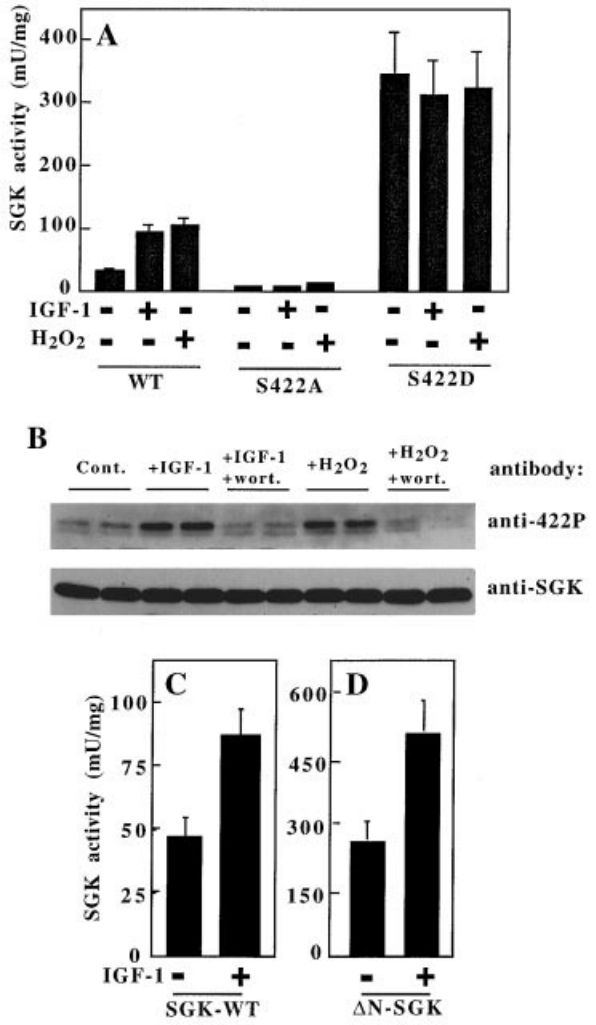

Figure 10 Effect of mutations at Ser^{422} on the activation of SGK in 293 cells

(**A**) 293 cells were transiently transfected with pEBG-SGK, pEBG-SGK[S422A] or pEBG-SGK[S422D] and then stimulated for 10 min with (+) or without (−) 50 ng/ml IGF-1 or for 25 min with (+) or without (−) 2 mM H_2O_2. The cells were lysed, and the GST–SGK was purified on glutathione–Sepharose (as in Figure 7) and assayed. Portions (50 μg) of cell-lysate protein were used for the assay of wild-type SGK and SGK[S422A]; 25-μg portions were used for SGK[S422D]. Each protein was expressed in similar amounts, as determined from SDS/PAGE followed by staining with Coomassie Blue (results not shown). (**B**) 293 cells were transfected with pEBG-SGK and stimulated with IGF-1 or H_2O_2, as in (**A**). The cells were lysed, and an aliquot of the extract (containing 30 μg of protein) was affinity-purified on glutathione–Sepharose as described in the legend to Figure 7, denatured in SDS, electrophoresed on an SDS/10% polyacrylamide gel and immunoblotted with an antibody raised against a phosphopeptide corresponding to the sequence surrounding Ser^{422} (anti-422P), as well as an antibody that recognizes both phosphorylated and dephosphorylated SGK equally well (anti-SGK) (see the Materials and methods section). (**C** and **D**) Same as in (**A**), except that the 293 cells were transiently transfected with pEBG-SGK (**C**) or pEBG-ΔN-SGK (**D**) as indicated, and stimulated for 10 min with 50 ng/ml IGF-1. Samples of cell lysate protein (50 μg) were used for the assays of wild-type SGK, and 10 μg was used for the assays of the N-terminally truncated enzyme.

and these residues and the sequences surrounding them are highly conserved in SGK (Figure 1), we investigated whether SGK could be activated by these and/or other signals in 293 cells.

Cells overexpressing GST–SGK were stimulated with IGF-1 or exposed to hydrogen peroxide, which induced a 2.5–4-fold activation (Figures 7A and 7B). Activation was strongly suppressed if the cells were first preincubated with the PtdIns 3-kinase inhibitor wortmannin (Figure 7B). In contrast, the immunosuppressant drug rapamycin, which prevents the activation of p70 S6 kinase, had no effect on the activation of SGK (results not shown).

The IGF-1 and hydrogen-peroxide-induced activation was caused by increased phosphorylation because, like the basal activity, the stimulated activity was abolished by treatment with PP2A (Figure 8). The fold-activation of GST–SGK induced by IGF-1 or hydrogen peroxide was slightly slower than activation of GST–PKBα under the same conditions (Figure 8).

The addition of PMA (400 ng/ml, for 30 min) or epidermal growth factor (EGF; 100 ng/ml for 30 min), which are much stronger activators of the classical mitogen-activated protein kinase cascade in 293 cells than IGF-1, did not induce any activation of SGK (results not shown).

Table 1 Comparison of the activity of SGK and PKBα towards synthetic peptides related to 'Crosstide'

The experiments were carried out with GST–ΔN-SGK and GST–PKBα purified from 293 cells and activated by PDK1. The activities are presented relative to peptide 1 (Crosstide). The concentration of each peptide was 30 μM. Amino acid substitutions are underlined. The serine residue in Crosstide that is phosphorylated by PKB is preceded by an asterisk.

Peptide	SGK	PKBα
1 GRPRTSS*FAEG	100%	100%
2 RPRTSSF	154	160
3 RPRTSAF	< 1	< 1
4 PRTSSF	< 1	< 1
5 RPRTSS	10	< 1
6 RPRTSTF	156	208
7 RPRAATF	73	55
8 KPRTSSF	2	6
9 RPKTSSF	49	26
10 RPRTSSF	154	160
11 RPRTSSL	135	69
12 RPRTSSV	131	67
13 RPRTSSA	107	26
14 RPRTSSK	146	48
15 RPRTSSE	84	21

Activation of SGK by co-transfection with PDK1 in 293 cells

SGK could be activated by co-transfection of its DNA with that of PDK1. The extent of activation increased with increasing amounts of PDK1 DNA, with about 4-fold activation at the highest levels of PDK1 DNA used. However, co-transfection did not increase the IGF-1-stimulated activation of SGK at any concentration of PDK1 DNA (Figure 9).

Evidence that the activation of SGK by IGF-1 and hydrogen peroxide involves the phosphorylation of Thr^{256} and Ser^{422}

The activity of SGK[T256A] was very low in unstimulated cells, and it could not be activated by either IGF-1 or hydrogen peroxide (Figure 7A), suggesting that the phosphorylation of Thr^{256} is essential for the activation of SGK by these stimuli. The activity of SGK[S422A] was also very low in unstimulated cells, and it could not be activated by IGF-1 or hydrogen peroxide (Figure 10A), suggesting that the phosphorylation of Ser^{422} is

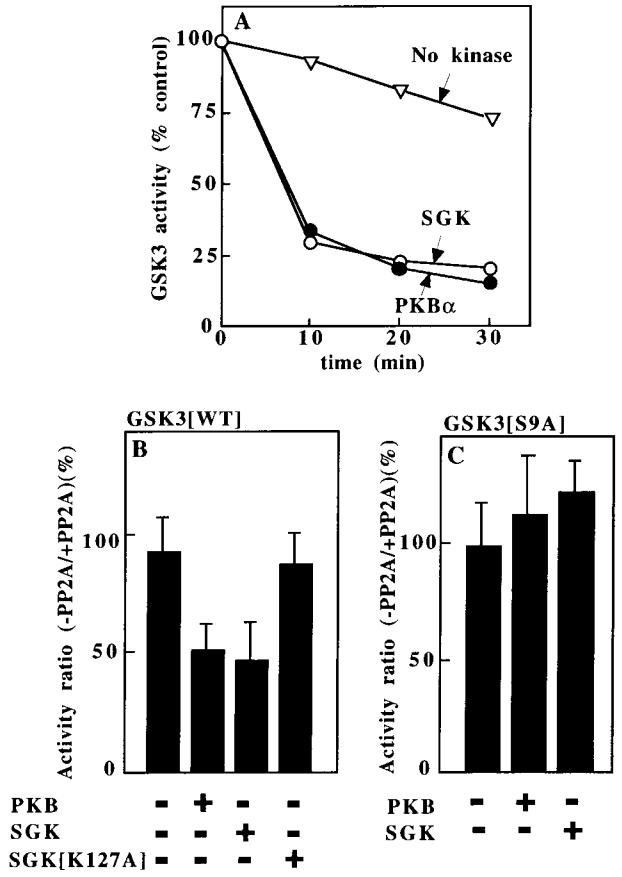

Figure 11 SGK and PKBα both inactivate GSK3 *in vitro* and in co-transfection experiments

(**A**) The incubations *in vitro* were performed as described for peptide phosphorylation, except that GSK3 (50 m-units) purified from rabbit skeletal muscle was used in place of the peptide. Units of GSK3 activity are defined as described in [31]. At various times after incubation with MgATP alone (▽), or in the presence of SGK (○) or PKBα (●), aliquots were removed and assayed for GSK3 activity as described in [31]. SGK and GSK3 were matched for activity towards Crosstide (0.3 unit/ml). (**B** and **C**) 293 cells *in vivo* were co-transfected with pCMV5 constructs expressing WT-EE-GSK3β or A9-Myc-GSK3β with or without pEBG-SGK, pEBG-SGK[K127A] (expressing catalytically inactive SGK) or pEBG-PKBα (6 μg per 6-cm dish). After transfection (24 h), the cells were lysed and GSK3 was assayed after immunoprecipitation using antibodies raised against the EE or Myc tags [27]. GSK3 activity was measured after incubation without or with 30 m-unit/ml PP2A to re-activate fully GSK3 [27]. GSK3 is expressed as a reactivation ratio, i.e. GSK3 activity measured without PP2A treatment divided by activity measured after PP2A treatment.

also essential for the activation of SGK. This was confirmed using a phospho-specific antibody (Figure 10B), which demonstrated that Ser^{422} phosphorylation increases in response to IGF-1 or hydrogen peroxide and that, with both stimuli, Ser^{422} phosphorylation is prevented by the PtdIns 3-kinase inhibitor wortmannin. The specificity of the antibody was demonstrated by the finding that it no longer recognized SGK after incubation with PP2A (results not shown). Incubation of the antibody with the phosphopeptide antigen (but not with the dephosphopeptide) also prevented recognition of SGK (results not shown).

Activation of SGK mutants by IGF1

The activity of GST–SGK[S422D] was high in 293 cells that had not been stimulated with IGF-1 (Figure 3) and, interestingly, was not activated further by IGF-1 (Figure 10A). Moreover, pre-incubation of 293 cells with 100 nM wortmannin (30 min) did not affect the high basal activity of SGK (results not shown). GST–ΔN-SGK was activated similarly to full-length wild-type SGK in response to IGF-1 (Figures 10C and 10D), indicating that the N-terminal non-catalytic domain is not required for activation in 293 cells.

Substrate specificity of SGK

We compared the substrate specificities of SGK and PKBα towards several synthetic peptides related to 'Crosstide' (Table 1). Like PKBα, SGK had an absolute requirement for the presence of an arginine residue five residues N-terminal to the site of phosphorylation, because mutation, even to another basic amino acid (Lys), almost abolished activity. The presence of an arginine three residues N-terminal to the site of phosphorylation was also important, but not quite as critical as for PKBα. Like PKBα, SGK required at least one residue C-terminal to the phosphorylation site, but the requirement for a bulky hydrophobic residue at this position was not as critical as for PKBα. Another significant difference between PKBα and SGK was that the latter was more tolerant of the substitution of serine by threonine at the site of phosphorylation. Although the results presented in Table 1 were obtained using PDK1-activated GST–ΔN SGK[S422D], similar results were also obtained with full-length SGK.

GSK3 is a protein thought to be a physiological substrate for PKB [31]. When PKBα and SGK were matched for activity towards 'Crosstide', both enzymes inactivated GSK3β at similar rates (Figure 11). GSK3 was also inactivated to a similar extent when co-expressed in 293 cells with either PKBα or SGK and, in both cases, the GSK3 could be reactivated with PP2A (Figure 11B). No inactivation occurred in these co-transfection exeriments when wild-type GSK3β was replaced by a GSK3 mutant in which the serine residue phosphorylated by PKB (Ser^9) was changed to Ala (Figure 11C). This indicates that the inactivation of GSK3 by SGK is also mediated via the phosphorylation of Ser^9.

DISCUSSION

Although it is well established that SGK is the product of an immediate-early gene whose levels increase within an hour in response to several agonists and pathological conditions (see the Introduction), the possibility that SGK is also regulated by reversible phosphorylation has not been investigated previously. In this paper, we establish that the activity of SGK expressed in 293 cells is determined by its level of phosphorylation (Figure 3), which increases within minutes in response to signals that activate PtdIns 3-kinase (Figures 7 and 8). We have also provided evidence that the key phosphorylation sites on SGK are Thr^{256} and Ser^{422}, the residues equivalent to those on PKBα that are targeted by PDK1 and PDK2 respectively.

SGK is phosphorylated and activated by PDK1 *in vitro* (Figure 4), and the major site of phosphorylation is Thr^{256} (Figure 6). Activation is abolished, and phosphorylation greatly decreased, if Thr^{256} is mutated to Ala (Figure 5). Thus the PDK1-induced activation of SGK is mediated by the phosphorylation of Thr^{256}. However, the effects of phosphorylation could not be mimicked by mutating Thr^{256} to an acidic residue, and such mutations actually decreased activity to the level of the PP2A-treated wild-type enzyme (Figure 3). This is similar to observations made with p70 S6 kinase [32], but is in contrast with PKBα, where mutation of Thr^{308} to Asp causes a partial activation of the enzyme [6]. The finding that phosphorylation of SGK by PDK1 is not totally abolished if Thr^{256} is mutated to Ala (Figure 5) indicates that PDK1 is capable of phosphorylating another site(s) on GST–

SGK[T256A] *in vitro*, but the identity of this site and its relevance (if any) to the activation process remain to be evaluated. The Thr^{308}Ala mutant of PKBα (but not the wild-type enzyme) is phosphorylated at Thr^{304} by PDK1 *in vitro* without causing any activation (D. R. Alessi, unpublished work from this laboratory).

Several lines of evidence suggest that one role for the phosphorylation of Ser^{422} is to accelerate phosphorylation at Thr^{256} by PDK1, and hence the activation of SGK. Thus GST–SGK is phosphorylated by PDK1 *in vitro* much more slowly than PKBα, but phosphorylation and activation are greatly potentiated by the mutation of Ser^{422} to Asp (Figure 5). The Ser^{422} to Asp mutation also greatly increases the activity of SGK expressed in 293 cells (Figures 3 and 10), and this results from increased phosphorylation (presumably at Thr^{256}) because it is abolished by treatment with PP2A (Figure 3), and because the Thr^{256}Ala/Ser^{422}Asp and Thr^{256}Asp/Ser^{422}Asp double mutants are inactive.

If it is assumed that the Ser^{422}Asp mutation mimics the effect of phosphorylation at this residue, and that the GST-fusion protein behaves similarly to wild-type SGK, then the following model for the activation of SGK would be consistent with the results obtained in this paper. (1) The PtdIns(3,4,5)P_3 generated in response to IGF-1 or hydrogen peroxide activates PDK2 (or a closely related enzyme), which then phosphorylates SGK at Ser^{422}. (2) The phosphorylation of Ser^{422} greatly potentiates the rate at which SGK is phosphorylated and activated by PDK1 (or a closely related enzyme). This model explains (i) why the IGF-1-induced or the hydrogen-peroxide-induced activation of SGK (Figure 8B) and its phosphorylation at Ser^{422} (Figure 10B) in 293 cells is suppressed by wortmannin (Figure 8B); (ii) why the activation of SGK by PDK1 *in vitro* is independent of PtdIns(3,4,5)P_3 (Figure 4); and (iii) why the activity of GST–SGK [S422D] is not suppressed by wortmannin. This model is similar to the mechanism proposed for the activation of p70 S6 kinase [15,16] but, in order to validate it, it will be necessary to find out whether PDK2 is indeed PtdIns(3,4,5)P_3-dependent, and to study the effect of PDK2 on the activation of SGK in the presence and absence of PDK1.

The mechanism of activation of SGK differs from that of PKB in several respects. First, the binding of PtdIns(3,4,5)P_3 to the PH domain of PKB is essential before any activation can take place [7]. Secondly, the interaction of PtdIns(3,4,5)P_3 with the PH domain of PKB causes its recruitment to the plasma membrane, which might facilitate its activation by membrane-associated PDK1 and PDK2. Thirdly, PDK1 greatly facilitates the PtdIns(3,4,5)P_3-dependent activation of PKB in lipid vesicles *in vitro*, and might therefore also be critical for the activation of PKB at the plasma membrane *in vivo*. These differences might explain why essentially no activation of PKB occurs *in vivo* until PtdIns(3,4,5)P_3 is elevated, and the larger and more rapid activation of PKB by IGF-1 or hydrogen peroxide in 293 cells (Figure 8). However, as for the activation of SGK, the activation of PKB might also be dependent on the interaction of PtdIns(3,4,5)P_3 with PDK2.

Like PKB and p70 S6 kinase, SGK phosphorylates peptides at serine or threonine residues that lie in sequences with arginine residues at positions $n-3$ and $n-5$, where n is the site of phosphorylation. The physiological substrates of SGK are unknown. However, like PKB, SGK is activated by a PtdIns 3-kinase-dependent pathway and (unlike p70 S6 kinase) activation is not prevented by the immunosuppressant drug rapamycin. Moreover, when matched for activity towards the standard peptide substrate 'Crosstide', SGK and PKBα catalysed the inactivation of GSK3 at similar rates *in vitro* (Figure 11A), and to similar extents in co-transfection experiments (Figure 11B). Since GSK3 is thought to be a physiological substrate of PKB [31], it is therefore possible that some of the physiological roles ascribed to PKB on the basis of overexpression of constitutively active mutants (reviewed in [2]) might actually be mediated by SGK. Since PKB and SGK might well be activated by the same protein kinases *in vivo* (PDK1 and PDK2), it might not be possible to distinguish their physiological roles by the use of dominant-negative mutants. The development of drugs that inhibit PKB, but not SGK, and vice versa, or the generation of mouse 'knock-outs', may be necessary to answer these questions.

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