# Characterization of the structure and regulation of two novel isoforms of serum- and glucocorticoid-induced protein kinase

Takayasu KOBAYASHI, Maria DEAK, Nick MORRICE and Philip COHEN[1]

MRC Protein Phosphorylation Unit, Department of Biochemistry, MSI/WTB Complex, Dow Street, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

The catalytic domain of serum- and glucocorticoid-induced protein kinase (SGK) is 54% identical with protein kinase B (PKB) and, like PKB, is activated *in vitro* by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and *in vivo* in response to signals that activate phosphatidylinositol (PI) 3-kinase. Here we identify two novel isoforms of SGK, termed SGK2 and SGK3, whose catalytic domains share 80% amino acid sequence identity with each other and with SGK (renamed SGK1). Like SGK1, the mRNA encoding SGK3 is expressed in all tissues examined, but SGK2 mRNA is only present at significant levels in liver, kidney and pancreas and, at lower levels, in the brain. The levels of SGK2 mRNA in H4IIE cells and SGK3 mRNA in Rat2 fibroblasts are not increased by stimulation with serum or dexamethasone, whereas the level of SGK1 mRNA is increased greatly. SGK2 and SGK3 are activated *in vitro* by PDK1, albeit more slowly than SGK1, and their activation is accompanied by the phosphorylation of $Thr^{193}$ and $Thr^{253}$ respectively, the residues equivalent to the Thr in the 'activation loop' of PKB that is targeted by PDK1. The PDK1-catalysed phosphorylation and activation of SGK2 and SGK3, like SGK1, is greatly potentiated by mutating $Ser^{356}$ and $Ser^{419}$ respectively to Asp, these residues being equivalent to the C-terminal phosphorylation site of PKB. Like SGK1, SGK2 and SGK3 are activated 5-fold via a phosphorylation mechanism when cells are exposed to $H_2O_2$ but, in contrast with SGK1, activation is only suppressed partially by inhibitors of PI 3-kinase. SGK2 and SGK3 are activated to a smaller extent by insulin-like growth factor-1 (2-fold) than SGK1 (5-fold). Like PKB and SGK1, SGK2 and SGK3 preferentially phosphorylate Ser and Thr residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr motifs.

Key words: glucocorticoid, IGF1, insulin, oxidative stress, PKB, SGK.

## INTRODUCTION

Protein kinase B (PKB, also called c-Akt) is activated *in vivo* by agonists that activate phosphatidylinositol (PI) 3-kinase and is thought to mediate many of the cellular effects of these signals, such as the insulin-induced activation of glycogen and protein synthesis and the protection of cells against apoptotic cell death (reviewed in [1,2]). The activation of PKB requires the phosphorylation of two sites, $Thr^{308}$ and $Ser^{473}$ [3]. 3-Phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylates $Thr^{308}$ *in vitro* [4,5], and can be converted into a form that phosphorylates $Ser^{473}$ as well as $Thr^{308}$ on interaction with a peptide corresponding to the C-terminal sequence of protein kinase C-related kinase-2 (PRK2) [6]. However, whether $Ser^{473}$ is phosphorylated *in vivo* by PDK1 remains to be established.

Residues equivalent to $Thr^{308}$ and $Ser^{473}$ are present in many protein kinases in the subfamily that includes cyclic AMP and cyclic GMP-dependent protein kinases and protein kinase C (AGC subfamily), such as p70 S6 kinase, and there is increasing evidence that PDK1 catalyses the activation of these enzymes *in vivo* [7–10]. However, the enzyme that is most similar to PKB (54% identity in the catalytic domain) is serum- and glucocorticoid-induced protein kinase (SGK). SGK is of additional interest because the level of its mRNA increases 5–10-fold upon exposure of Rat2 fibroblasts to serum or glucocorticoids [11]. SGK mRNA and protein are also induced in response to other stimuli, such as follicle-stimulating hormone [12], high extracellular osmolarity [13], injury of the brain [14,15], aldosterone [16] and transfection with p53 [17]. However, like PKB, SGK is also activated within minutes via a phosphorylation mechanism when cells are stimulated with insulin, insulin-like growth factor-1 (IGF1) or serum, or exposed to oxidative stress [18,19]. As observed with PKB, activation by these stimuli is prevented by inhibitors of PI 3-kinase and is accompanied by the phosphorylation of $Thr^{256}$ and $Ser^{422}$, the residues equivalent to $Thr^{308}$ and $Ser^{473}$ of PKB [18,19], SGK is phosphorylated at $Thr^{256}$ by PDK1 *in vitro* [18,19], and phosphorylation is greatly enhanced by the mutation of $Ser^{422}$ to Asp [18], which presumably mimics the effect of phosphorylation by introducing a negative charge [18].

Here we identify two novel isoforms of SGK, termed SGK2 and SGK3, which are the products of distinct genes, and compare their tissue distribution, induction by serum and glucocorticoids, activation by phosphorylation and substrate specificity with SGK (hereafter termed SGK1). Despite, their high degree of sequence similarity, the mechanisms that regulate the level and activity of SGK2 and SGK3 differ significantly from those that regulate SGK1 in several respects.

Abbreviations used: SGK, serum- and glucocorticoid-induced protein kinase; PKB, protein kinase B; PDK1, 3-phosphoinositide-dependent protein kinase-1; PRK2, protein kinase C-related protein kinase-2; PI, phosphatidylinositol; IGF1, insulin-like growth factor-1; GSK3, glycogen synthase kinase-3; GST, glutathione S-transferase; PP2A, protein phosphatase 2A; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; FBS, foetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; NCBI, National Centre for Biotechnology Information; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of flight.

[1] To whom correspondence should be addressed (e-mail pcohen@bad.dundee.ac.uk).

© 1999 Biochemical Society

## EXPERIMENTAL

### Materials

Human PDK1 was expressed as a glutathione S-transferase (GST) fusion protein in 293 cells and purified by affinity chromatography on glutathione–Sepharose [5]. Dexamethasone, wortmannin and LY 294002 were purchased from Sigma (Poole, Dorset, U.K.). All peptides were synthesized by Dr. Graham Blumberg (Department of Biochemistry, University of Bristol, Bristol, U.K.). Expression plasmids of SGK1 and an N-terminally truncated form of SGK1 (ΔN-SGK1[61–431]) were identical with pEBG-SGK and pEBG-ΔN-SGK[61–431] described in [18]. The pEBG-2T-2 vector was made by insertion with 5′-GAT CTC GGA TCC ACT AAC GGT AC-3′ and 5′-CGT TAG TGG ATC CGA-3′ between *Bam*HI and *Kpn*I sites of pEBG-2T. This introduced a new *Bam*HI site which was located in a different reading frame from the original pEBG-2T vector. The protease-inhibitor cocktail was from Roche Diagnostics (Lewes, East Sussex, U.K.). Protein phosphatase 2A (PP2A) purified from bovine heart was provided by Dr. R. MacKintosh in this Unit.

### 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. Buffer B comprised 50 mM Tris/HCl, pH 7.5, 1 mM EGTA and 0.1 % (v/v) 2-mercaptoethanol. Buffer C comprised 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1 mM EGTA, 0.5 mM dithiothreitol and 1 mg/ml BSA. Cell-lysis buffer was Buffer A containing 1 $\mu$M microcystin-LR, 0.2 mM PMSF and protease-inhibitor cocktail (one tablet/500 ml).

### Cloning of SGK isoforms

All the expressed sequence tags (ESTs) were obtained from the IMAGE consortium (U.K. HGMP Resource Centre, Cambridge, U.K.). A full-length clone encoding human SGK2 was obtained as follows. Analysis of EST AA130828 showed that it encoded a novel kinase which was homologous with human SGK1. A 5′ rapid amplification of cDNA ends (5′-RACE) was then carried out using human kidney cDNA (Marathon-Ready cDNA; Clontech, Pato Alto, CA, U.S.A.) as a template, since there was no stop codon upstream of the first ATG. The primers used for 5′-RACE of SGK2 were 5′-GCT CTG GAC TTG GGG TCC CAG CTG GGC-3′, 5′-GTT GAT GTT CCC ATT GGC CCT GGA GGG-3′ and 5′-GCT GGG CAT TTG GGT TGG CTG AAG GCC-3′. Fragments of 200–220 bp were obtained in all three cases, and the PCR products were cloned into the pCR-2.1-TOPO vector (Invitrogen, San Diego, CA, U.S.A.). Eight clones out of the ten clones analysed possessed a 200-bp fragment, and sequence analysis showed that these corresponded to the 5′ portion of EST AA130828. We designated this protein kinase SGK2$\alpha$. Two other clones had longer fragments (500 and 600 bp). Sequence analysis showed that these clones corresponded to another form of SGK2 with an additional 60 amino acid residues at the N-terminus and a different 5′ non-coding region. This enzyme was designated SGK2$\beta$. A mouse clone (accession number AI 386362) was also identified by interrogating the National Centre for Biotechnology Information (NCBI) EST database with the sequence of human SGK2$\alpha$ that was predicted to encode a full-length protein of mouse SGK2$\alpha$. We also assembled the full-length murine cDNA using the clones AA790370 (which encodes the first 1240 nucleotides) and AA138663 [that starts from nucleotide 1140 and terminates in the poly(A)tail].

A full-length clone encoding human SGK3 was obtained as follows. Analysis of EST AA219166 showed that it encoded the partial sequence of a novel protein kinase which had homology with SGK1 and SGK2. Since this cDNA contained a gap in the central portion, a PCR reaction was carried out using a human skeletal-muscle library as a template. The primers used for the PCR were 5′-AACATCCGTTTTTGGTTGGATTGC-3′ and 5′-GGGTAGATGTTAGTGTAAAC-3′. Since this clone did not contain an initiating ATG codon, 5′-RACE was carried out using human kidney cDNA as a template. The primers used for 5′-RACE were 5′-ATA AAG TTC TGG ATA CCT AAC TAG G-3′, 5′-GAA GGA ATG CTC TGA CAT CTG GAT GG-3′ and 5′-GAT CTG ACT GGT GTT TTG GAC TGT CC-3′. Unlike the 5′ RACE of SGK2, which showed sharp bands of PCR products when analysed on an agarose gel, that of SGK3 produced a smear. We therefore ligated these heterogeneous products into a pCR-2.1-TOPO vector (Invitrogen) and analysed the 20 clones obtained. Sequence analysis revealed that 12 clones contained the missing part of the new SGK sequence, which we called SGK3.

In order to introduce a *Bam*HI site at the 5′-end in-frame with the GST sequence of the pEBG-2T-2 vector, and a *Not*I site at the 3′-end of each open reading frame, PCR amplification was performed using the following primers: SGK2$\alpha$: 5′-GGA TCC AGC TGC CTG ATC ATT GCT AC-3′ and 5-GCG GCC GCC TAG CAA TCC AAG ATG TCA TC-3; SGK2$\beta$: 5′-GGA TCC CAG GGG TTG CTT ACC TCG GG-3′ and 5′-GCG GCC GCC TAG CAA TCC AAG ATG TCA TC-3′; full-length SGK3: 5′-GGA TCC AAG CCC TGA AGA AGA TTC CTG CC-3′ and 5′-GCG GCC GCT CAC AAA AAT AAG TCT TC-3′; SGK3 lacking the N-terminal 52 residues (ΔN-SGK3[53–429]): 5′-GGA TCC TGG ACA GTC CAA AAC ACC AG-3′ and 5′-GCG GCC GCT CAC AAA AAT AAG TCT TC-3′. The PCR products were cloned into a pCR-2.1-TOPO vector (Invitrogen) and sequenced. Each fragment was digested with *Bam*HI and *Not*I and inserted into the cloning site of pEBG-2T-2. $Ser^{356}$ of SGK2$\alpha$ and $Ser^{419}$ of SGK3 were mutated to Asp by *in vitro* mutagenesis using PCR.

### Expression of SGK isoforms in 293 cells

Each 10 cm- and 6 cm-diameter dish of cells was transfected with 10 $\mu$g or 3 $\mu$g respectively of the pEBG-SGK constructs using a modified calcium phosphate method [3]. At 24 h after transfection the cells were deprived of serum for 16 h, then stimulated in one of the following ways: 50 ng/ml IGF-1 (10 min), 400 ng/ml PMA (30 min), 10 % (v/v) foetal bovine serum (FBS) (30 min), 1 $\mu$M dexamethasone (60 min), 2 mM $H_2O_2$ (25 min), 5 $\mu$g/ml anisomycin (30 min), 0.4 M sorbitol (30 min), 30 min of hypo-osmotic stress [3 ml of Dulbecco's modified Eagle's medium) (DMEM) diluted to 4 ml with water] and UV-C irradiation (200 J/m$^2$). After each treatment, the cells were lysed and the activity of SGK isoforms was measured after affinity purification of the GST-fusion proteins on glutathione–Sepharose, as described previously for SGK1 [18].

### Dephosphorylation, phosphorylation and assay of SGK isoforms *in vitro*

Each GST–SGK isoform (0.4 $\mu$g) was incubated for 20 min at 30 °C with protein phosphatase 2A (PP2A; 30 m-units/ml) in a 10 $\mu$l incubation containing 20 mM Tris/HCl, pH 7.5, 0.1 %

© 1999 Biochemical Society

(v/v) 2-mercaptoethanol, 0.1 mM EGTA and 1 mg/ml BSA. After addition of microcystin-LR to 1 $\mu$M to inactivate PP2A, SGK (0.4 $\mu$g) was phosphorylated for 30 min at 30 °C in a mixture (20 $\mu$l) containing 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 % (v/v) 2-mercaptoethanol, 0.1 mM EGTA, GST–PDK1 (0.03–300 ng) diluted in Buffer C and 100 $\mu$M unlabelled ATP. SGK assays were initiated by the addition of 30 $\mu$l of a mixture containing 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 % (v/v) 2-mercaptoethanol, 0.1 mM EGTA, 100 $\mu$M [$\gamma$-$^{32}$P]ATP (500 c.p.m./pmol), 50 $\mu$M of the peptide substrate Crosstide (GRPRTSSFAEG) and 4.2 $\mu$M of residues 5–24 of the specific protein inhibitor of cAMP-dependent protein kinase. After 10 min at 30 °C, the reactions were terminated by spotting aliquots on to P81 phosphocellulose paper, followed by immediate immersion in 75 mM phosphoric acid. The papers were washed, dried and analysed [3]. One unit of SGK was that amount which phosphorylated 1 nmol of Crosstide in 1 min.

### Preparation of RNA and Northern blotting

Rat2 fibroblasts or H4IIE hepatoma cells ($10^6$ cells) were plated on to 10 cm-diameter dishes and incubated for 24 h in DMEM containing 10 % FBS. The medium was changed to DMEM without serum and the lls incubated for a further 24 h. After stimulation with 10 % FBS and 1 $\mu$M dexamethasone, total RNA was isolated from the cells using an RNeasy Mini kit (Qiagen, Crawley, West Sussex, U.K.). RNA (5 $\mu$g) was electrophoresed through 1.2 %-agarose gels in the presence of 0.67 M formaldehyde, transferred to Hybond-N+ (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) and cross-linked under UV light (UV Stratalinker; Stratagene, La Jolla, CA, U.S.A.). $^{32}$P-labelled probes were generated by labelling the open reading frames of each SGK isoform with [$\alpha$-$^{32}$P]dATP using a Multiprime DNA labelling kit (Amersham Pharmacia Biotech). Hybridization was performed in Rapid-hyb buffer (Amersham Pharmacia Biotech) for 2 h at 65 °C. Membranes were washed with 2 × SSC/0.1 % SDS for 30 min at room temperature and then for 15 min at 65 °C, then washed with 0.2 × SSC/0.1 % SDS for 15 min at 65 °C and exposed to X-ray film (Hyperfilm, Amersham) at −70 °C for 3–5 days (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate).

## RESULTS

### Cloning of novel SGK isoforms

SGK was originally identified as a glucocorticoid- and osmotic-stress-responsive gene (see the Introduction). Searching for new SGK isoforms, the NCBI database was interrogated with the sequence of SGK. This search identified two human ESTs that showed considerable similarity to the original gene. Sequence analysis of these ESTs revealed that they represent two new isoforms of the SGK gene family that we term SGK2 and SGK3. The original SGK isoform is therefore termed SGK1.

As none of these ESTs were full-length clones, 5′-RACE and PCR reactions were carried out to obtain the missing parts of the sequence (see the Experimental section). This led to the identification of two splice variants of SGK2, termed SGK2$\alpha$ and SGK2$\beta$ (Figure 1). The DDB/EMBL/GenBank® accession number for human SGK2$\alpha$ and SGK2$\beta$ are AF169034 and AF186470 respectively. SGK2$\alpha$ encodes a protein of 367 residues with a calculated molecular mass of 41.1 kDa. SGK2$\beta$ encodes a protein of 427 amino acids with a calculated molecular mass of 47.6 kDa. An EST encoding murine SGK2$\alpha$ was also identified (Figure 1). Unlike human SGK2$\alpha$, the murine cDNA contains

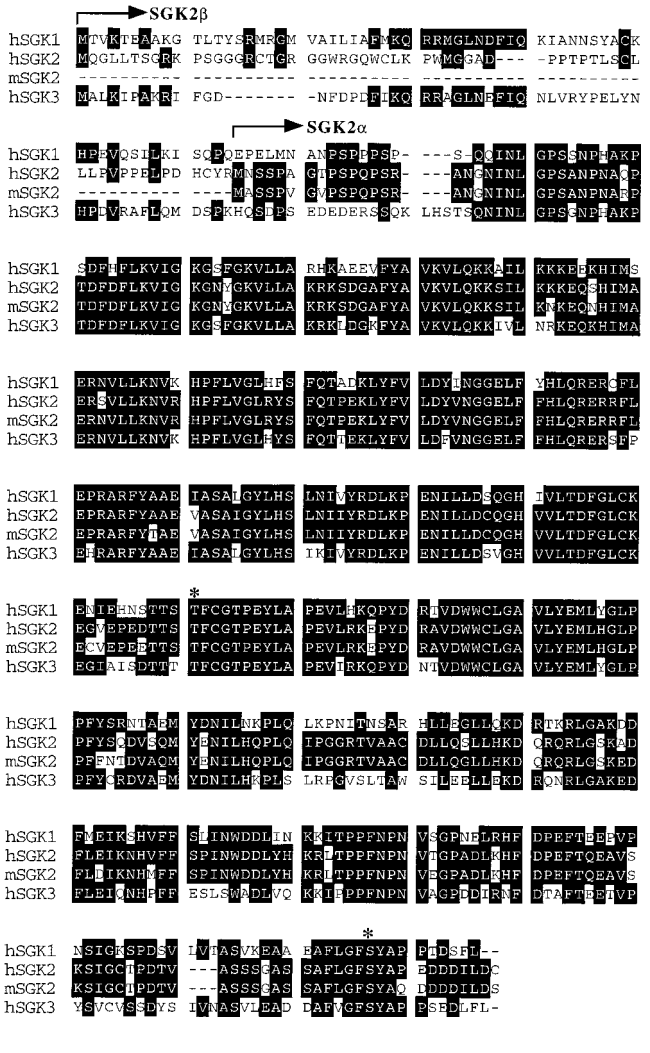


**Figure 1 Alignment of amino acid sequences of SGK isoforms**

The alignment of human (h) SGK1, hSGK2, murine (m) SGK2 and hSGK3 was carried out using the Clustal W program [24]. Identities are shaded in black and the initiation codons of SGK2$\alpha$ and SGK2$\beta$ are indicated by arrows. The two key phosphorylation sites are marked with asterisks. The DDB/EMBL/GenBank® accession number for murine SGK2 is AF169033.

an in-frame stop codon immediately preceding the initiating Met. This establishes that the shortest splice variant does indeed start at this position.

The nucleotide sequence of SGK3 contained two in-frame ATG codons (DDB/EMBL/GenBank® accession number AF169035). The sequence surrounding the second ATG conforms more closely to the consensus that frequently surrounds initiation codons. Moreover, a stop codon (TAA) is present immediately upstream of the first ATG. For these reasons, the site of initiation is assumed to be the second ATG. This would encode a 429-residue 49.0 kDa protein. If the first ATG is used for initiation, it would generate a polypeptide 62 amino acids longer.

The amino acid sequences of the three SGK isoforms are about 80 % identical with one another in the catalytic domain, whereas the short C-terminal non-catalytic domains are less similar (44–68 % identity). The N-terminal 85 residues that precede the catalytic domain are much less similar. In this region there is only about 25 % identity between SGK1 and SGK3

© 1999 Biochemical Society

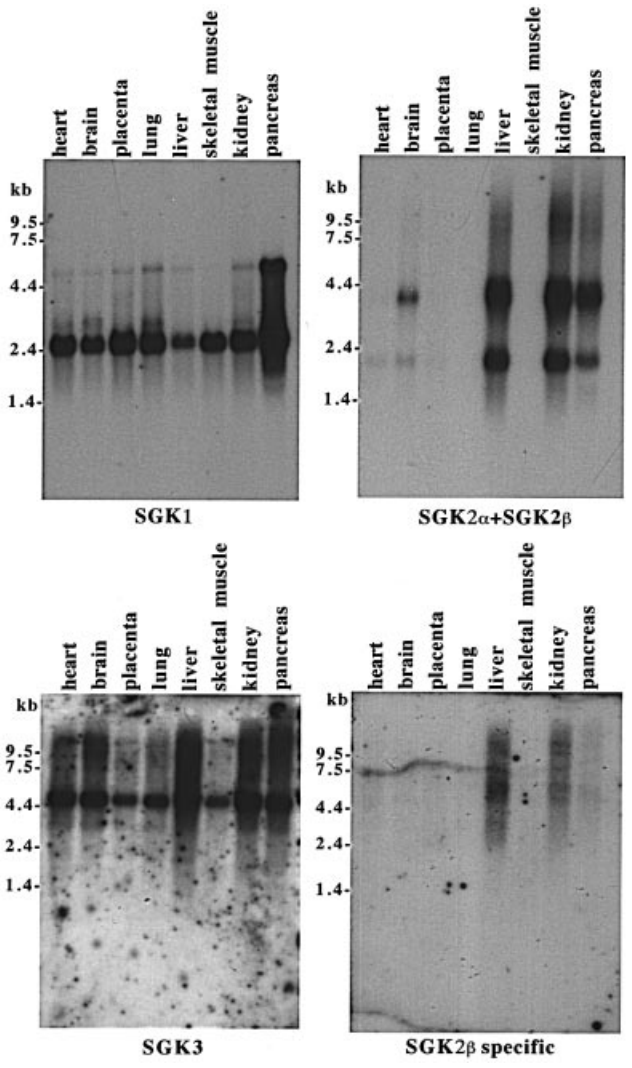


**Figure 2 Expression of the mRNA encoding SGK1, SGK2 and SGK3 in different human tissues**

The Northern blots containing 2 $\mu$g of RNA were hybridized with $^{32}$P-labelled SGK1, SGK2 and SGK3 cDNA probes. The membranes were then exposed to X-ray film for 3–5 days. The probe for SGK2 is common to sGK2$\alpha$ and SGK2$\beta$. The result obtained with a specific SGK2$\beta$ probe is shown in a separate panel. The positions of standard RNA markers of defined sizes in kb are marked.

and almost no identity between SGK2 and the other isoforms (Figure 1).

### Tissue distribution of SGK-isoform mRNAs

As reported previously by others [13], the level of SGK1 mRNA was similar in all tissues tested, with a major 2.6 kb transcript and a minor 7 kb transcript. The highest levels of both transcripts were in the pancreas. The level of SGK3 mRNA was also similar in each tissue, with a major 5 kb transcript in each case. In contrast, mRNA which hybridizes with a probe that recognizes SGK2$\alpha$ and SGK2$\beta$ had a much more restricted tissue distribution, 2.1 kb and 4.0 kb transcripts being present in liver, kidney, pancreas and brain (Figure 2). An SGK2$\beta$-specific probe also recognized transcripts in liver and kidney, but the levels were much lower and the signal was rather diffuse.

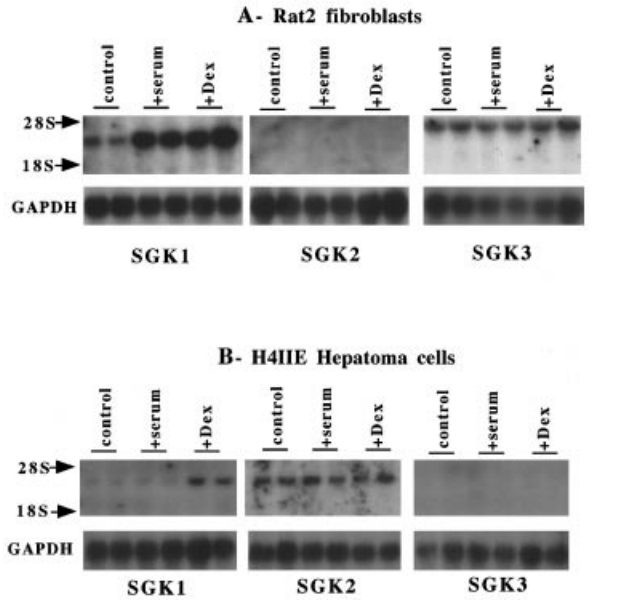


**Figure 3 Induction of the mRNA encoding SGK isoforms by different agonists**

(**A**) Rat2 fibroblasts that had been deprived of serum for 24 h were incubated for 2 h with 10% FBS (serum) or 1 $\mu$M dexamethasone (Dex) or with no further additions (control). Total RNA extracted from each dish of cells (5 $\mu$g) was subjected to electrophoresis and analysed for SGK isoform transcripts by Northern blotting. To demonstrate equivalent loading of the samples, the membranes were re-probed with $^{32}$P-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA (bottom panel). The positions of the standard RNA markers 18 S and 28 S ribosomal RNA are indicated. (**B**) Same experiment as (**A**), but using H4IIE hepatoma cells.

### Effect of serum and dexamethasone on the level of SGK-isoform mRNA in Rat2 fibroblasts and H4IIE hepatoma cells

SGK1 is an immediate early gene and its mRNA increases greatly within 1 h of stimulation with serum and glucocorticoids in several cell lines [11]. We therefore examined the effects of these stimuli on mRNA encoding all three SGK isoforms. In Rat2 fibroblasts, SGK1 mRNA was strongly induced by serum or dexamethasone after 2 h as expected, whereas the level of SGK3 mRNA was not affected by these agonists. SGK2 mRNA was not expressed in Rat2 fibroblasts (Figure 3A). In rat H4IIE hepatoma cells, dexamethasone, but not serum, induced the formation of SGK1 mRNA, whereas the level of SGK2 mRNA was unaffected by either agonist. SGK3 mRNA was not expressed in H4IIE cells (Figure 3B).

### Expression of SGK isoforms in 293 cells

All three isoforms were expressed as GST-fusion proteins in 293 cells. The sizes of GST–SGK2$\alpha$ (70 kDa), GST–SGK3 (75 kDa) and GST–$\Delta$N-SGK3 (70 kDa) were consistent with their calculated molecular masses (Figure 4). However, nearly all the GST–SGK2$\beta$ was expressed as free GST (25 kDa), presumably due to proteolysis of the full-length fusion protein (Figure 4, lane 3). Deletion of the N-terminal region of SGK3 improved its level of expression (Figure 4, lanes 5 and 6), although not to the same extent as SGK1 (Figure 4, lanes 1 and 2). Typically 400, 100 and 150 $\mu$g of 90%-pure GST–$\Delta$N-SGK1[61–431], 80% pure GST–SGK2$\alpha$ and 90% pure GST–$\Delta$N-SGK3[53–429] respectively were obtained from ten 10 cm-diameter dishes of cells.

© 1999 Biochemical Society

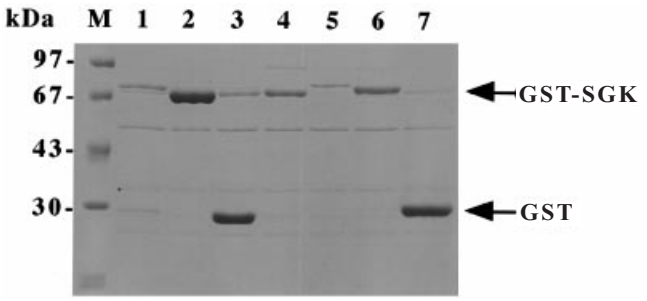


**Figure 4 Expression of SGK isoforms as GST-fusion proteins in 293 cells**

GST-fusion proteins were expressed in 293 cells and purified from 200 $\mu$g of cell lysate protein by chromatography on glutathione–Sepharose. Proteins eluted with 15 mM glutathione were subjected to electrophoresis on a SDS/10%-polyacrylamide gel and stained with Coomassie Blue. Cells were transfected with following constructs: lane 1, pEBG-SGK1 expressing full-length SGK1; lane 2, pEBG-$\Delta$N-SGK1 expressing an N-terminally truncated form of SGK1; lane 3, pEBG-SGK2$\beta$ expressing full-length SGK2$\beta$; lane 4, pEBG-SGK2$\alpha$ expressing full-length SGK2$\alpha$; lane 5, pEBG-SGK3 expressing full-length SGK3; lane 6, pEBG-$\Delta$N-SGK3 expressing an N-terminally truncated form of SGK3; lane 7, pEBG-2T expressing GST. The marker proteins (lane M) and their molecular masses are also shown.

### Phosphorylation and activation of SGK isoforms by PDK1

The two key phosphorylation sites corresponding to residues Thr$^{308}$ and Ser$^{473}$ of PKB$\alpha$ [3] are conserved in SGK2 (Thr$^{193}$ and Ser$^{356}$ in SGK2$\alpha$) and SGK3 (Thr$^{253}$ and Ser$^{419}$), as well as SGK1 (Thr$^{256}$ and Ser$^{422}$). We [18] and others [19] have shown that PDK1 activates wild-type SGK1 *in vitro* by phosphorylating Thr$^{256}$ (see also Figures 5A and 5D). Wild-type SGK2$\alpha$ and SGK3 were also activated by PDK1 *in vitro*, although higher concentrations of PDK1 were required for activation (Figures 5B and 5C) and phosphorylation (Figures 5E and 5F), especially with SGK2. Maximal activation of all SGK isoforms was observed after incubation for 30 min with 1.5 $\mu$g/ml PDK1 (Figures 5A, 5B and 5C). At this concentration of PDK1 the stoichiometries of phosphorylation were close to 1 mol/mol for SGK1 and SGK3 and 0.2 mol/mol for SGK2 (results not shown). If the PDK1 concentration was increased 10-fold, the level of phosphorylation of SGK2 and SGK3 increased considerably, but without any further activation (Figures 5D, 5E and 5F). This suggested that PDK1 is capable of phosphorylating other sites on SGK2 and SGK3 that do not affect activity directly.

We have demonstrated that an SGK1 mutant in which Ser$^{422}$ (the PDK2 site) is mutated to Asp has a specific activity more than 10-fold higher than that of the wild-type enzyme after

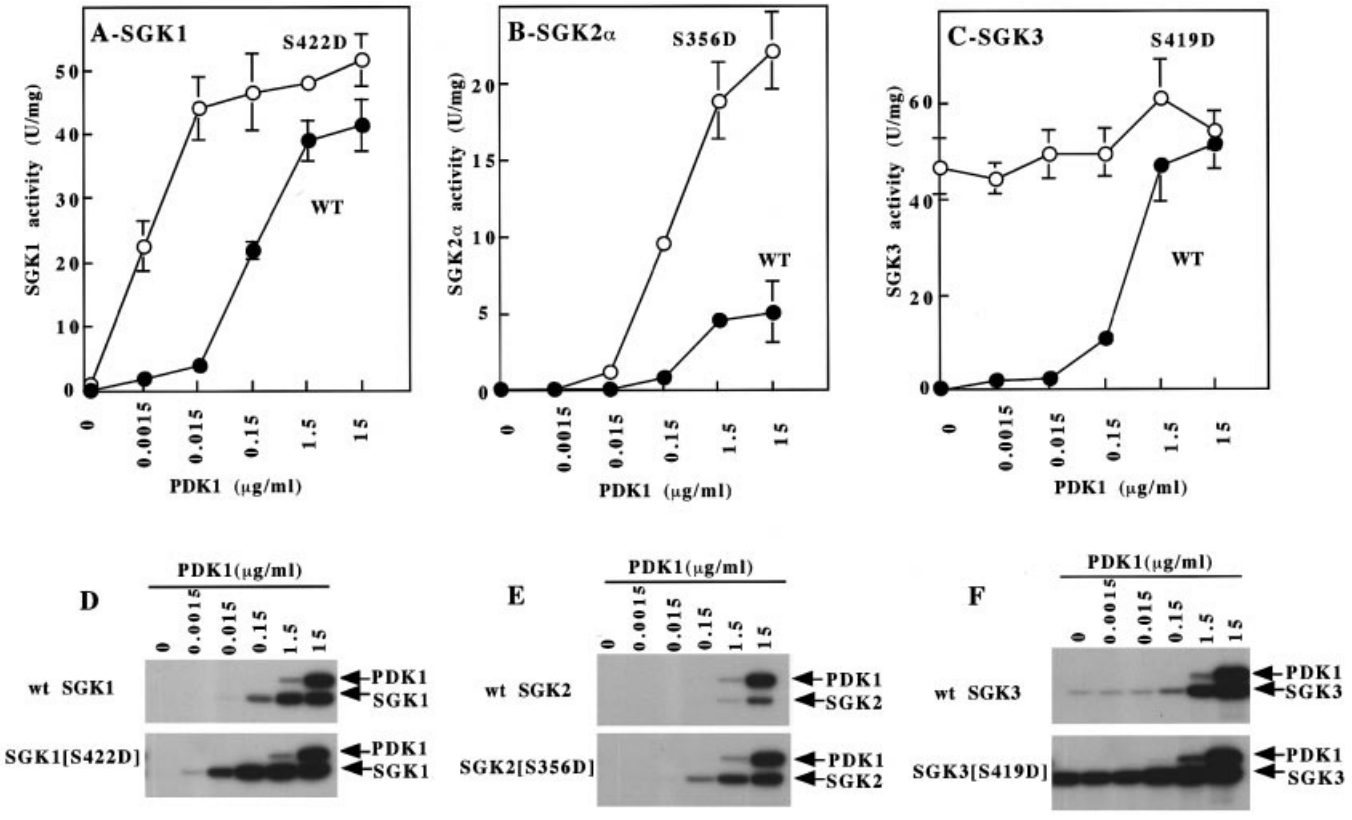


**Figure 5 Phosphorylation and activation of wild-type and mutant SGK isoforms by PDK1**

(**A**), (**B**) and (**C**) Purified GST-fusion proteins (0.4 $\mu$g) were incubated with PP2A as described in the Experimental section, and microcystin-LR (1 $\mu$M) was then added to inactivate PP2A. The SGK isoforms (20 $\mu$g/ml) were then phosphorylated using the indicated amounts of purified GST-PDK1. After 30 min at 30 °C, SGK activity was measured using Crosstide as substrate. The results are shown as means $\pm$ S.D. for three experiments. The closed circles (●) show the results with wild-type SGKs and the open circles (○) results with mutant SGKs in which the residue equivalent to Ser$^{473}$ of PKB had been changed to Asp. (**D**), (**E**) and (**F**) Same as (**A**), (**B** and (**C**), except that 100 $\mu$M [$\mu$-$^{32}$P]ATP (500 c.p.m./pmol) was used instead of unlabelled ATP. The reactions were 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 for 5 min at 95 °C, electrophoresis on SDS/10% polyacrylamide gels and autoradiography. The upper panels show results with wild-type (wt) SGK isoforms and the lower panels results with mutant SGKs. The positions of each SGK isoform and PDK1 on the gels are marked.

© 1999 Biochemical Society

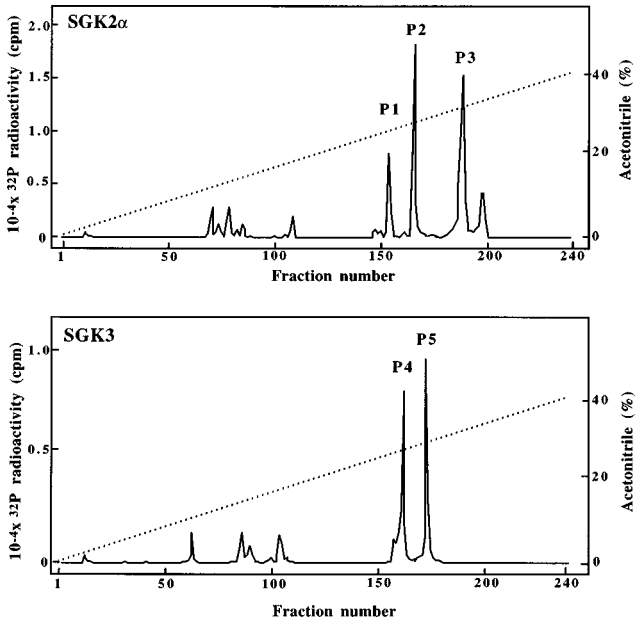


**Figure 6 Separation of tryptic phosphopeptides from SGK2α and SGK3 after phosphorylation by PDK1**

GST–SGK2α (upper panel) or GST–SGK3 (lower panel) were inactivated by treatment with PP2A, phosphorylated with PDK1 (1.5 μg/ml) and Mg[γ-$^{32}$P]ATP (5000 c.p.m./pmol), denatured in SDS, alkylated with 4-vinylpyridine and subjected to SDS/PAGE as described in the legend to Figure 7. The bands corresponding to SGK2α and SGK3 were excised, eluted from the gel, precipitated with trichloroacetic acid and digested with trypsin as described previously [25]. The digest was applied to a Vydac $C_{18}$ column equilibrated in 0.1% trifluoroacetic acid and the $^{32}$P-labelled peptides (P) resolved using a linear acetontirile gradient in 0.1% trifluroacetic acid at a flow rate of 0.8 ml/min. Fractions (0.4 ml each) were collected and analysed for $^{32}$P radioactivity (continuous line). The acetonitrile gradient is shown by the diagonal broken line.

**Table 1 Comparison of the activity of SGK isoforms toward synthetic peptides related to Crosstide**

The experiments were carried out with GST–ΔN-SGK1[Ser422Asp], GST–SGK2α[Ser356Asp] and GST–ΔN-SGK3[Ser419Asp] purified from 293 cells. The activities are presented relative to peptide 1 (Crosstide). The concentration of each peptide was 30 μM. Amino acid substitutions to peptide 2 are underlined. Results shown are the averages for two experiments each agreeing within ±10%.

| | | | Activity (%) | | |
|---|---|---|---|---|---|
| No. | Peptide | Isoform ... | SGK1 | SGK2 | SGK3 |
| 1 | GRPRTSSFAEG | | 100 | 100 | 100 |
| 2 | RPRTSSF | | 135 | 182 | 137 |
| 3 | RPRTSAF | | < 1 | < 1 | < 1 |
| 4 | PRTSSF | | < 1 | < 1 | < 1 |
| 5 | RPRTSS | | 5.1 | 8 | 18 |
| 6 | RPRTSTF | | 129 | 192 | 156 |
| 7 | RPRAATF | | 44 | 61 | 56 |
| 8 | KPRTSSF | | < 1 | < 1 | < 1 |
| 9 | RPKTSSF | | 11 | 8 | 48 |
| 10 | RPRTSSL | | 108 | 185 | 113 |
| 11 | RPRTSSV | | 90 | 124 | 139 |
| 12 | RPRTSSA | | 49 | 82 | 123 |
| 13 | RPRTSSK | | 84 | 128 | 200 |
| 14 | RPRTSSE | | 28 | 34 | 67 |

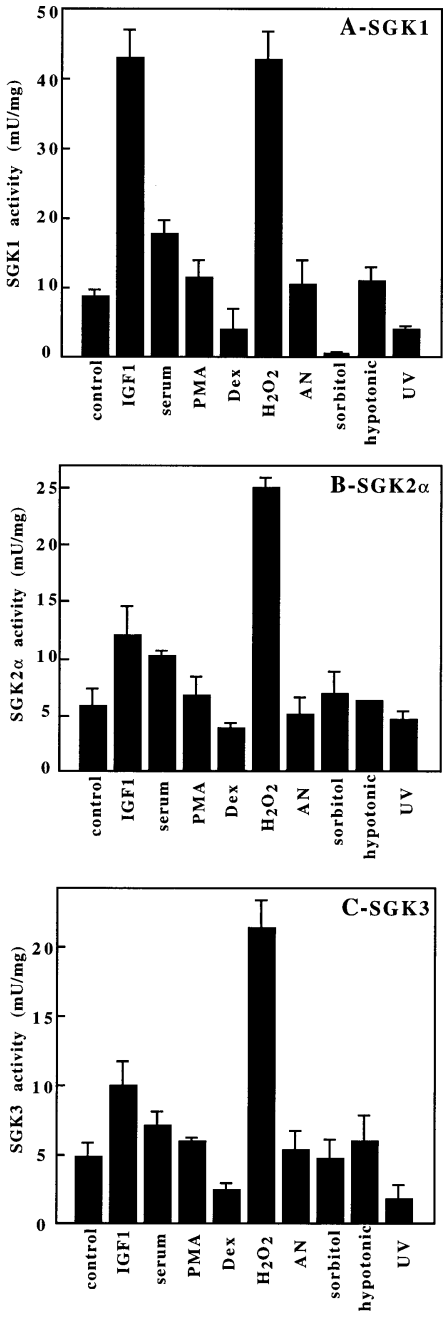


**Figure 7 Activation of SGK isoforms in 293 cells**

Cells were transiently transfected with DNA constructs expressing (**A**) GST–SGK1, (**B**) GST–SGK2α and (**C**) GST–SGK3. At 24 h after transfection, the cells were deprived of serum for 16 h, then exposed to 50 ng/ml IGF1 (10 min), 400 ng/ml PMA (30 min), 10% FBS (serum) (30 min), 1 μM dexamethasone (Dex, 60 min), 2 mM $H_2O_2$ (25 min), 5 μg/ml anisomycin (AN, 30 min), 0.4 M sorbitol (30 min), hypotonic stress (1 ml of water added to 3 ml of DMEM, 30 min), UV radiation (30 min after exposure to 200 J/m$^2$) or left untreated (control). After each treatment, the cells were lysed in ice-cold lysis buffer and SGK activity in 100 μg of cell lysate protein was measured. The expression of each isoform was similar in each transfection as judged by SDS/PAGE followed by staining with Coomassie Blue (results not shown). Results are means ± S.D. for three separate experiments.

expression in 293 cells [18]. This high basal activity results from increased phosphorylation at Thr$^{256}$ and can be abolished by treatment with PP2A [18]. In the present study we found that PP2A-treated SGK1[Ser422Asp] was re-activated (Figure 5A)

© 1999 Biochemical Society

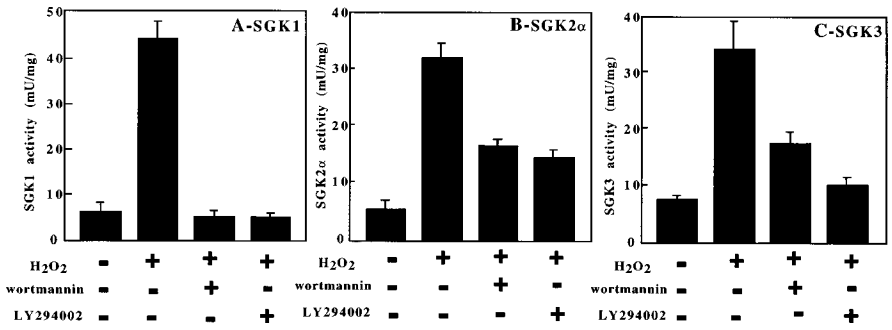


**Figure 8 Effect of PI 3-kinase inhibitors on the activation of SGK isoforms by $H_2O_2$ in 293 cells**

Cells were transiently transfected with DNA constructs expressing (**A**) GST–SGK1, (**B**) GST–SGK2$\alpha$ and (**C**) GST–SGK3. At 24 h after transfection, the cells were deprived of serum for 16 h. The transfectants were then pretreated for 10 min with 100 nM wortmannin or for 1 h with 100 $\mu$M LY294002 and then stimulated for 25 min with 2 mM $H_2O_2$. Results are means $\pm$ S.D. for three separate experiments.

and phosphorylated (Figure 5D) at a much higher rate than PP2A-treated wild-type SGK1.

The corresponding mutants of SGK2 and SGK3 in which the Ser residues equivalent to $Ser^{422}$ of SGK1 were mutated to Asp SGK2[Ser356Asp] and SGK3[Ser419Asp]) also had high activities after expression in 293 cells (results not shown). After treatment with PP2A, SGK2$\alpha$ was inactivated and, as for SGK1, SGK2[Ser356Asp] was activated (Figure 5B) and phosphorylated (Figure 5E) by PDK1 much more effectively than wild-type SGK2$\alpha$. In contrast, SGK3[Ser419Asp] could not be inactivated by PP2A, and incubation with PDK1 and MgATP did not increase the activity further (Figure 5C). SGK3[Ser419Asp] did, however, become phosphorylated on incubation with MgATP, even in the absence of PDK1 (Figure 5F), presumably as a result of autophosphorylation. Phosphorylation did not appear to be catalysed by PDK1 present as a contaminant, because no PDK1 could be detected in the preparation by immunoblotting (results not shown).

The SGK2[Ser356Asp] mutant and wild-type SGK3 were incubated for 30 min at 30 °C using 1.5 $\mu$g/ml PDK1 and MgATP, which led to the incorporation of 1.2 and 1.3 mol of phosphate per mol of protein respectively. The reactions were terminated, the mixtures digested with trypsin and phosphopeptides produced chromatographed on a $C_{18}$ column as described in [18] (Figure 6). The major phosphothreonine-containing peptides from the SGK2 and SGK3 digests (peptides P2 and P5 in Figure 6A and 6B) were subdigested with Asp-N proteinase and subjected to matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS and solid-phase Edman sequencing [20] to identify the sites of phosphorylation. These experiments revealed that the phosphopeptide from SGK2 corresponded to residues 189–207 phosphorylated at $Thr^{193}$, whereas the SGK3 phosphopeptide corresponded to residues 249–267 phosphorylated at $Thr^{253}$. These experiments establish that PDK1 does indeed phosphorylate SGK2 and SGK3 at sites equivalent to $Thr^{256}$ of SGK1.

The phosphoserine-containing tryptic peptides P1, P3 and P4 (Figure 6) were also analysed by MALDI-TOF MS and Edman sequencing. These experiments revealed that peptide P1 corresponded to residues 277–287 phosphorylated at $Ser^{279}$, while peptide P3 comprised residues 334–367 phosphorylated at $Ser^{334}$. Peptide P4 corresponded to residues 73–99 and was phosphorylated at either $Ser^{75}$ or $Ser^{77}$; the latter was the major site of phosphorylation. These findings are considered further in the Discussion section.

The activation of SGK2 and SGK3 by PDK1 was unaffected by lipid vesicles containing phosphatidylserine and phosphatidyl choline [4] and slightly inhibited by the further inclusion of 10 $\mu$M PI 3,4,5-trisphosphate (results not shown). These results are similar to those obtained previously with SGK1.

### Substrate specificities of SGK isoforms

It has been shown previously that SGK1 and PKB have similar specificities towards a panel of synthetic peptides, preferentially phosphorylating Ser and Thr residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr motifs [18]. In the present study, the specificity requirements of SGK2$\alpha$ and SGK3 were also found to be similar (Table 1), although SGK3 appears to tolerate the presence of a Lys instead of an Arg at position $n-3$ (where $n$ is the site of phosphorylation) a little better than SGK1 or SGK2$\alpha$ (Table 1).

### Activation of SGK isoforms in 293 cells

Each SGK isoform was expressed in 293 cells, and their ability to be activated acutely by a variety of stimuli was examined. As reported previously [18,19], stimulation with IGF1 induced a 4–5-fold increase in SGK1 activity (Figure 7), which reached a maximum after 10 min (results not shown). In contrast, SGK2$\alpha$ and SGK3 were only activated 2 fold by IGF1 (Figure 7). Activation was maximal after 5 min and sustained for at least 20 min (results not shown). The agonist which produced the strongest activation of SGK2$\alpha$ and SGK3 (5-fold) was $H_2O_2$, a finding similar to that observed with SGK1 (Figure 7). Serum was also a weak activator of all three isoforms. However, osmotic shock (treatment with 0.4 M sorbitol), which potently inhibited SGK1, had no effect on the activity of SGK2$\alpha$ or SGK3 (Figure 7).

The activation of SGK2 and SGK3 induced by $H_2O_2$ was only inhibited partially when the cells were preincubated with wortmannin or LY 294002 (two inhibitors of PI 3-kinase) prior to stimulation with $H_2O_2$. In contrast, the activation of SGK1 was abolished (Figure 8).

## DISCUSSION

In this paper we identify two novel isoforms of SGK1, termed SGK2 and SGK3, which share 80 % identity with SGK1 in their catalytic domains. Nevertheless, despite this similarity, SGK2

© 1999 Biochemical Society

and SGK3 differ significantly from each other, and from SGK1, in a number of respects. First, the mRNAs encoding SGK1 and SGK3 are widely expressed, but that encoding SGK2 has a more restricted distribution, being highly expressed in liver, kidney and pancreas and, at lower levels, in the brain (Figure 2). Secondly, SGK2 mRNA and SGK3 mRNA are not increased by cell stimulation with serum or glucocorticoids (Figure 3), whereas SGK1 mRNA levels increase greatly within 1 h of exposure to these agonists ([11]; Figure 3).

SGK2 and SGK3 are activated by PDK1 *in vitro*, albeit more slowly than SGK1 (Figure 5), and activation is accompanied by the phosphorylation of the same residue in the 'activation loop' between kinase subdomains VII and VIII ($Thr^{256}$ in SGK1, $Thr^{193}$ in SGK2 and $Thr^{253}$ in SGK3). Moreover, like SGK1 [18] and p70 S6 kinase [9], the PDK1-catalysed phosphorylation and activation of SGK2 and SGK3 *in vitro* is greatly potentiated by mutation to Asp of the C-terminal Ser ($Ser^{422}$ in SGK1, $Ser^{356}$ in SGK2 and $Ser^{419}$ in SGK3), suggesting that phosphorylation of this residue is a prerequisite for the PDK1-catalysed phosphorylation of the activation-loop Thr. These observations raise the question of the identity of the protein kinase(s) that phosphorylates SGK isoforms at the C-terminal Ser. PDK1 is converted into a form that phosphorylates PKB at $Ser^{473}$, as well as $Thr^{308}$, by interaction with a peptide corresponding to the C-terminal sequence of PRK2 [6]. However, this same peptide actually prevents PDK1 from phosphorylating SGK1 (T. Kobayashi, unpublished work) and p70 S6 kinase [21]. Whether PDK1 can be converted into a form that phosphorylates SGK isoforms at both sites by interaction with a different protein, or whether SGK isoforms are phosphorylated at this residue *in vivo* by a distinct protein kinase, remains to be established.

In contrast with SGK1, which is phosphorylated by PDK1 at one major site ($Thr^{256}$ [18]), SGK2 and SGK3 become phosphorylated at two additional Ser residues when incubated with PDK1 and MgATP (Figure 6). SGK2 is phosphorylated at $Ser^{279}$ and $Ser^{334}$, as well as $Thr^{193}$. The sequence surrounding $Ser^{334}$ (Ser-Ile-Gly-Cys-Thr-Pro-Asp-Thr-Val-Ala) resembles that surrounding $Thr^{193}$ (Thr-Phe-Cys-Gly-Thr-Pro-Glu-Tyr-Leu-Ala) (Figure 3), suggesting that $Ser^{334}$ is phosphorylated by PDK1 and raising the possibility that physiological substrates of PDK1 might include proteins that are not kinases. Ser residues equivalent to $Ser^{334}$ are present in SGK1 and SGK3 (Figure 1), but are not phosphorylated significantly by PDK1 [18] (Figure 6). The reason for this is unclear, but may be related to the particular amino acid replacements in the vicinity of this residue.

Interestingly, $Ser^{279}$ lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser sequence (Figure 1) which conforms to the optimal consensus for phosphorylation by SGK and PKB [18,22]. Thus $Ser^{279}$ phosphorylation is likely to be an autophosphorylation event catalysed by SGK2 itself after it has been activated by PDK1. This finding also raises the intriguing possibility that $Ser^{279}$ may be phosphorylated by another protein kinase *in vivo*, such as another SGK isoform or PKB. It will clearly be of considerable interest to examine whether $Ser^{279}$ phosphorylation occurs in cells and tissues and whether it alters the catalytic and regulatory properties of SGK2. $Ser^{279}$ is not conserved in either SGK1 or SGK3 (Figure 1).

SGK3 also became phosphorylated at two Ser residues ($Ser^{77}$ and $Ser^{79}$) upon incubation with PDK1 and MgATP, which are not conserved in SGK1 or SGK2 (Figure 1). Although these residues do not lie in optimal consensus sequences for either SGK or PDK1, they are probably autophosphorylation events catalysed by SGK3 for three reasons. First, activated SGK3 autophosphorylates in the absence of PDK1 (Figure 5F); secondly, the SGK3 concentration in these incubations is very high compared with those used with optimal peptide substrates; thirdly, basic amino acids ($Lys^{74}$ and $His^{76}$) are present three residues N-terminal to $Ser^{77}$ and $Ser^{79}$, which is one of the prerequisites for phosphorylation by SGK.

The response of SGK2 and SGK3 to agonists also differs in several respects from SGK1. For example, SGK2 and SGK3 are activated by IGF1 to a smaller extent (although just as rapidly) than SGK1 (Figure 7). The activation in response to $H_2O_2$ is similar for all three isoforms (5-fold), but inhibitors of PI 3-kinase only suppress the activation of SGK2 and SGK3 partially, whereas the activation of SGK1 is abolished (Figure 8). In addition, SGK2 and SGK3 activities are unaffected by exposure to osmotic stress, whereas SGK1 (Figure 7) and PKB [23] activities are virtually abolished. Understanding the basis for these differences will require further experimentation. It will also be of interest to compare the subcellular localization of SGK2 and SGK3 with that of SGK1 and to see whether, like SGK1 [19], these isoforms translocate to the nucleus or another organelle(s) in response to different agonists. A definitive answer to these questions will require the development of isoform-specific antibodies sufficiently sensitive to detect the endogenous levels of SGK2 and SGK3.

A number of physiological roles have been ascribed to PKB based on results obtained by overexpression of constitutively active mutants (reviewed in [1,2]). However, SGK isoforms have a similar specificity to PKB (Table 1) and are activated *in vivo* by the same stimuli. Moreover, SGK1 can mimic the ability of PKB to inactivate glycogen synthase kinase 3 *in vitro* and in co-transfection experiments [18]. It is therefore possible that some physiological roles thought to be performed by PKB are mediated by an SGK isoform. Conversely, activation of the epithelial sodium channel produced by co-expression with SGK1 in *Xenopus* oocytes [16] may really mediated by another SGK isoform or by PKB.

We thank the Novartis Foundation (Japan) for the Promotion of Science for the award of a Postdoctoral Fellowship to (to T.K.). This work was supported by the U.K. Medical Research Council, The Royal Society of London, The British Diabetic Association, the Louis Jeantet Foundation, AstraZeneca, NovoNordisk, Pfizer and SmithKline Beecham.

## REFERENCES

1 Alessi, D. R. and Cohen, P. (1998) Curr. Opin. Genet. Dev. **8**, 55–62
2 Cohen, P. (1999) Philos. Trans. R. Soc. London B **354**, 485–495
3 Alessi, D. R., Andjelkovic, M., Caudwell, F. B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. A. (1996) EMBO J. **15**, 6541–6551
4 Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B. and Cohen, P. (1997) Curr. Biol. **7**, 261–269
5 Alessi, D. R., Deak, M., Casamayor, A., Caudwell, F. B., Morrice, N., Norman, D. G., Gaffney, P., Reese, C. B., MacDougall, C. N., Harbison, D. et al. (1997) Curr. Biol. **7**, 776–789
6 Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P. and Alessi, D. R. (1999) Curr. Biol. **9**, 393–404
7 Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. and Parker, P. J. (1998) Science **281**, 2042–2045
8 Dutil, E. M., Toker, A. and Newton, A. C. (1998) Curr. Biol. **8**, 1366–1375
9 Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N. and Avruch, J. (1998) Curr. Biol. **8**, 69–81
10 Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C. and Hemmings, B. A. (1998) Science **279**, 710–714
11 Webster, M. K., Goyas, L. and Firestone, G. L. (1993) J. Biol. Chem. **268**, 11482–114485
12 Richards, J. S., Fitzpatrick, S. L., Clemens, J. W., Morris, J. K., Alliston, T. and Sirois, J. (1995) Hormone Res. **50**, 223–255
13 Waldegger, S, Barth, P., Raber, G. and Lang, F. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 4440–4445

© 1999 Biochemical Society

14 Hollister, R. D., Page, K. J. and Hyman, B. T. (1997) Neurosci. Lett. **79**, 1111–1119
15 Imaizumi, K., Tsuda, M., Wanaka, A., Tohyama, M. and Takagi, T. (1994) Mol. Brain Res. **26**, 189–196
16 Naray-Fejes-Toth, Canessa, C., Cleaveland, E. S., Aldrich, G. and Fejes-Toth, G. (1999) J. Biol. Chem. **274**, 16973–16978
17 Maiyar, A. C., Hunag, A. J., Phu, P. T., Cha, H. H. and Firestone, G. L. (1996) J. Biol. Chem. **271**, 12414–14222
18 Kobayashi, T. and Cohen, P. (1999) Biochem. J. **339**, 319–328
19 Park, J., Leong, M. L. L., Buse, P., Maiyar, A. C., Firestone, G. L. and Hemmings, B. A. (1999) EMBO J. **18**, 3024–3033
20 Morrice, N. and Powis, S. J. (1998) Curr. Biol. **8**, 713–716
21 Balendran, A., Currie, R., Armstrong, C., Avruch, J. and Alessi, D. R. (1999) J. Biol. Chem., in the press
22 Alessi, D. R., Caudwell, F. B., Andjelkovic, M., Hemmings, B. A. and Cohen, P. (1996) FEBS Lett. **399**, 333–338
23 Meier, R., Thelen, M. and Hemmings, B. A. (1998) EMBO J. **17**, 7294–7303
24 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) Nucleic Acids Res. **22**, 4673–4680
25 Cuenda, A., Alonso, G., Morrice, N., Jones, M., Meier, R., Cohen, P. and Nebreda, A. (1996) EMBO J. **15**, 4156–4164

Received 23 July 1999/6 September 1999; accepted 13 September 1999

© 1999 Biochemical Society