Activation of SGK1 by HGF, Rac1 and integrin-mediated cell adhesion in MDCK cells: PI-3K-dependent and -independent pathways

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Summary

The SGK1 protein belongs to the AGC gene family of kinases that are regulated by phosphorylation mediated by PDK1. SGK1 regulation is accomplished by several pathways including growth-factor and stress-mediated signaling. We have expanded the analysis of SGK1 regulation in epithelial cells. We used HA-tagged SGK1 to transiently transfect MDCK cells and study the regulation of SGK1 upon stimulation with HGF, cAMP or upon adhesion of the cells to immobilized fibronectin. In addition, we studied the regulation of SGK1 activity by small GTP-binding proteins of the Rho family.

Treatment of MDCK cells with HGF leads to a time-dependent activation of SGK1 that is blocked by wortmanin. This activation requires the conserved phosphorylation site present in the activation loop of the kinase (T256 in SGK1) and the phosphorylation site present in a hydrophobic domain at its C-terminus (S422 in SGK1), which are targets for PDK1/PDK2-mediated regulation of SGK1. We tested whether SGK1 could be activated by cAMP as it contains a putative PKA site. We were unable to demonstrate a significant activation of HA-SGK1 by cAMP stimulation under conditions where we detect cAMP-mediated phosphorylation of the transcription factor CREB.

Cotransfection of SGK1 with activated small GTP-binding proteins revealed that Rac1, but not Rho or Rap1, induces activation of SGK1. However, this activation was wortmanin insensitive and dominant-negative Rac1 did not inhibit the HGF-mediated activation of SGK1. Adhesion of MDCK cells to immobilized fibronectin also leads to activation of SGK1. However, it appears that the integrin-mediated activation of HA-SGK1 differs from AKT activation in the fact that AKT phosphorylation was blocked by wortmanin (or LY294002) whereas HA-SGK1 was not. The adhesion-dependent activation, however, requires the intact phosphorylation sites of SGK1. Co-transfection of HA-SGK1 with RacV12 results in increased activity in adherent cells compared with HA-SGK1 alone. Since RacN17 failed to inhibit adhesion dependent-activation of SGK1, it suggests that integrin activation is achieved by a parallel Rac-independent pathway.

The activation of SGK1 by HGF and integrin provides a link between HGF-mediated protection of MDCK from de-attachment induced apoptosis (anoikis). We demonstrate that dephosphorylation of the transcription factor FKRHL1 induced by cell de-attachment is prevented by activated SGK1, suggesting that SGK1 regulates cell survival pathways.

In summary, we demonstrate that SGK1 activation could be achieved through signaling pathways involved in the regulation of cell survival, cell-cell and cell-matrix interactions. SGK1 activation can be accomplished via HGF, PI-3K-dependent pathways and by integrin-mediated, PI-3K independent pathways. In addition, activation of SGK1 by the small GTP-binding protein Rac1 has been observed.

Key words: Adhesion, Kinases, MDCK, HGF

Introduction

The serum- and glucocorticoid-inducible kinases (SGK1,2,3) are serine/threonine kinases that belong to the AGC (protein kinase A; protein kinase G and protein kinase C) subfamily that includes cAMP and cGMP dependent kinases, PKC and AKT (Webster et al., 1993a; Webster et al., 1993b). The expression of SGK1, but not of SGK2 or SGK3, is acutely regulated by glucocorticoids and serum (Kobayashi et al., 1999). SGK1 has been proposed to participate in the regulation of epithelial sodium channels (Alvarez de la Rosa et al., 1999; Chen et al., 1999). The expression of SGK mRNA has been shown to be regulated by a SP-1 regulatory element (Alliston et al., 1997) and p53-mediated transcriptional activation (Maiyar et al., 1996), in addition to being mediated by glucocorticoids.

The post-transcriptional regulation of SGK has recently begun to be described. It has been shown that the subcellular distribution of SGK is subjected to both cell cycle and hormonal regulation that correlates with the phosphorylation state of the protein (Buse et al., 1999), suggesting that SGK expression and activity serves as an integration point for several signaling pathways. However, the molecular mechanism that leads to regulation of SGK activity has not been fully characterized. The structure of SGK shares significant homology with PKA, PKB/AKT, PKC and p70S6

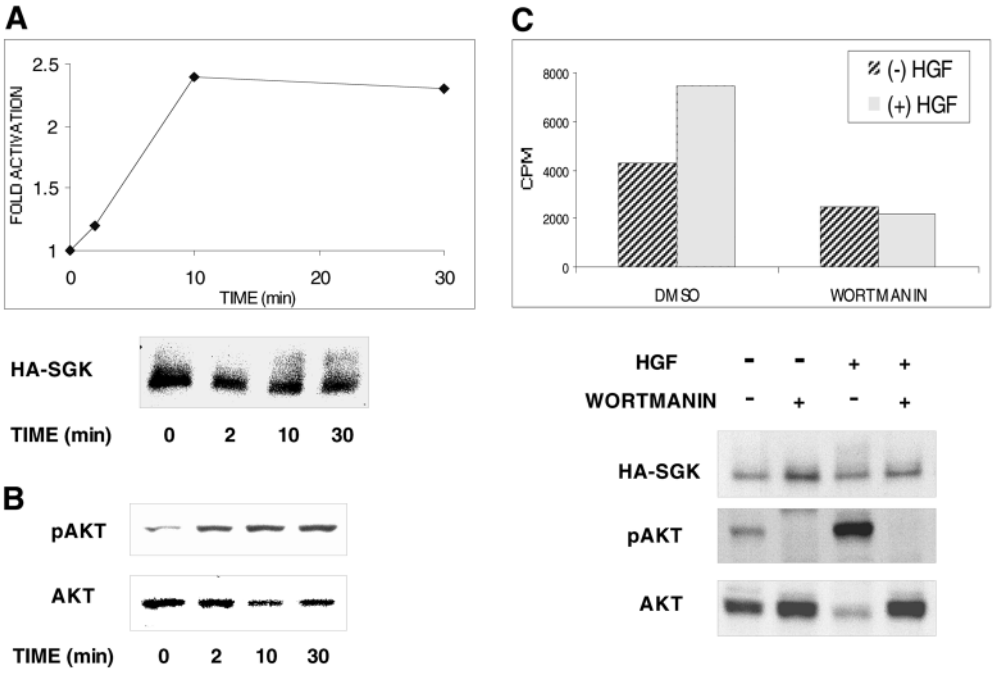

Fig. 1. Activation of SGK1 by HGF is wortmanin dependent. (A) Time course of SGK1 activation by HGF. MDCK cells were transiently transfected with HA-SGK1, stimulated with HGF and at the indicated times, cells were harvested and HA-SGK1 activity was measured as described in the Materials and Methods. The lower insert depicts the amount of HA-SGK1 present in the assay at the indicated times. (B) Time course activation of AKT by HGF. Cell extracts prepared as in (A) were separated in a SDS-PAGE and analyzed for the presence of phosphorylated AKT (T473)(pAKT) or total AKT as described in the Materials and Methods. (C) HGF activation of HA-SGK1 or AKT is blocked by wortmanin. MDCK cells were transfected with HA-SGK1, stimulated with HGF for 10 minutes in the presence or absence (DMSO) of wortmanin, and the kinase activity associated with HA-SGK1 was measured as in (A). The lower insert depicts the amount of phosphorylated AKT (T473)(pAKT), total AKT and HA-SGK1 present under the conditions described. Despite HGF-treated extract being under-loaded in the AKT gel, it contains detectable levels of pAKT.

kinase (Webster et al., 1993a; Webster et al., 1993b). All of these kinases are subjected to covalent modification by 3-phosphoinositide-dependent kinase 1, PKD1 (Alessi et al., 1998; Cheng et al., 1998; Le Good et al., 1998; Pullen et al., 1998; Vanhaesebroeck and Alessi, 2000), where the conserved phosphorylation site present in the activation loop of these kinases (T256 in SGK1) is phosphorylated by PDK1 (Alessi et al., 1998; Cheng et al., 1998; Le Good et al., 1998; Pullen et al., 1998; Vanhaesebroeck and Alessi, 2000). In addition, a phosphorylation site present in a hydrophobic domain at its C-terminus (S422 in SGK1), the so-called PDK2 site, is also conserved. The activity of SGK is therefore regulated by the phosphorylation of these two sites.

Although it is well established that the phosphorylation of SGK at T256 in the activation loop is carried out by PDK1, the kinase(s) involved in the phosphorylation of the site at S422 is still poorly defined. The regulation of AKT via the PDK2 site has been described as accomplished either by a ‘modified’ PDK1 (Balendran et al., 1999), an autophosphorylation event (Toker and Newton, 2000), or directly (Delcommenne et al., 1998) or indirectly (Lynch et al., 1999) by the integrin-linked kinase, ILK. It remains to be determined whether a similar process regulates SGK activity. However, it is known that phosphorylation of the S422 is required for maximal activation of SGK by enhancing phosphorylation of the activation loop residue T256 (Kobayashi and Cohen, 1999; Kobayashi et al., 1999; Park et al., 1999). Regulation of SGK activity by extracellular stimuli, including insulin, insulin-like growth factor, serum or oxidative stress (Kobayashi and Cohen, 1999; Park et al., 1999) has been described. On the basis of these studies carried out by Kobayashi and Cohen (Kobayashi and Cohen, 1999), a model for the activation of SGK has been proposed that involves (1) IGF-1-induced production of PIP3 that activates a PDK2-like enzyme leading to phosphorylation of S422 and (2) PDK1 phosphorylation of T256. This model is an analog of the proposed regulation of p70S6k (Alessi et al., 1998; Pullen et al., 1998) and represents an example of a phosphorylation-dependent substrate for PDK1 (Vanhaesebroeck and Alessi, 2000). Since SGK activity has been described as being regulated by extracellular stimulation that leads to PI-3K activation, we have investigated whether SGK activation could be achieved through signaling pathways involved in the regulation of cell-cell and cell-matrix interactions. We demonstrate that in the epithelial cell line MDCK, SGK activation can be accomplished via a scatter-factor-mediated PI-3K-dependent pathway and by an integrin-mediated PI-3K independent pathway. In addition, activation of SGK by the small GTP-binding protein Rac1 has been observed.

Materials and Methods

cDNA constructs

Human SGK1 (accession number Y10032) cDNA was obtained by PCR using forward 5′ primer (5′ GGA TCC GTG ATG ACG GTG AAA ACT GAG G 3′) and reverse primer 3′ primer (5′ GAA TTC TCA GAG GAA AGA GTC CGT GGG AG 3), TAQ polymerase (Life Technologies, Rockville, MD) and human placental cDNA (Clontech, Palo Alto, CA) as the template. The PCR product was directly ligated into the vector PCR 2.1 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and transformed into DH5alpha bacteria (Life Technologies). Point mutation constructs (K127A, T256A, T369A, S422A and S422D) were created using primers designed with specific nucleic acid mutations and the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. All SGK1 sequences were confirmed by an on-site sequence core facility using an automatic sequencer. Both

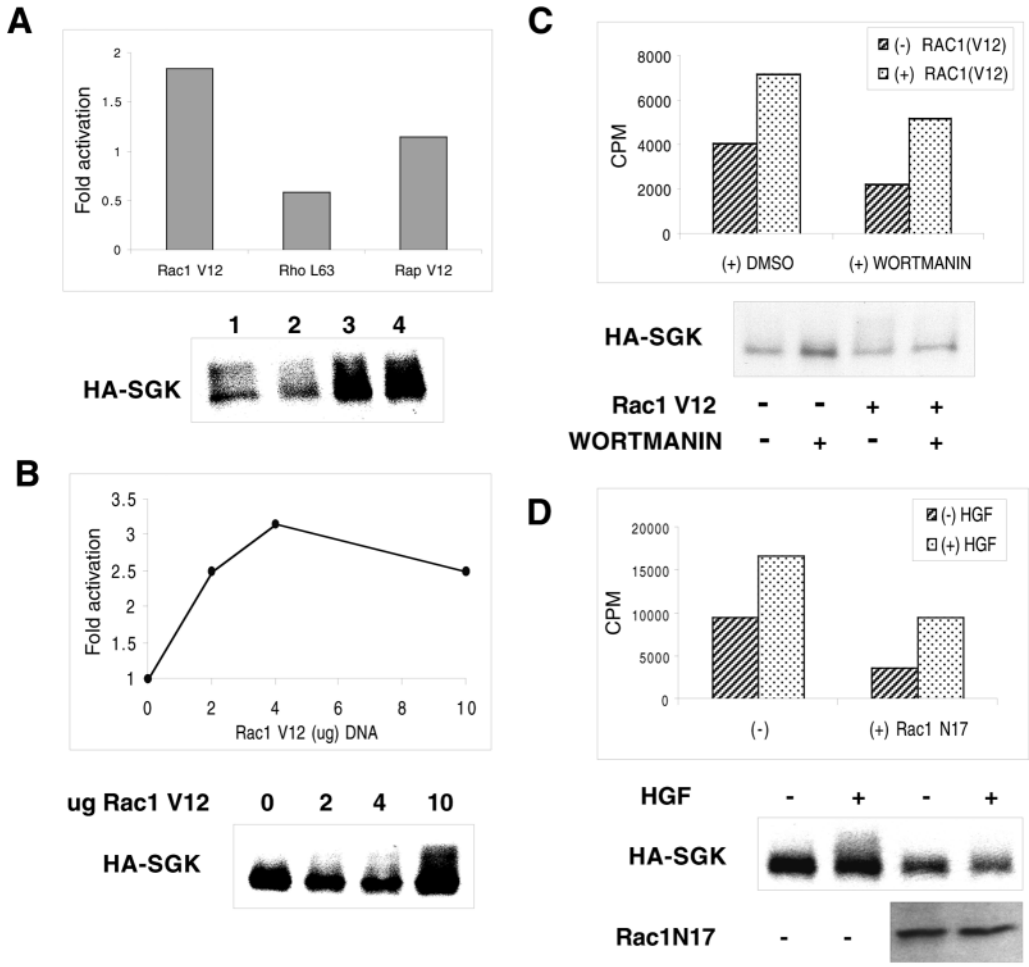

Fig. 2. Activation of HA-SGK1 by Rac1V12 is wortmanin independent. (A) MDCK cells were co-transfected with HA-SGK1 and either Rac1V12 (lane 1), RhoL63 (lane 2), Rap1V12 (lane3) or HA-SGK alone (lane 4), and the kinase activity associated with HA-SGK1 was assayed as described in the Materials and Methods. The results are presented as the fold increase in activation over the activity obtained in the absence of the small GTP-binding proteins. The lower insert depicts the amount of HA-SGK1 present in the assay. (B) Dose-dependent activation of HA-SGK1 by Rac1V12. MDCK cells were cotransfected with HA-SGK1, and the indicated amounts of Rac1V12 and the kinase activity was assayed as described in the Materials and Methods. The lower insert depicts the amount of HA-SGK1 present in the assay. (C) The effects of wortmanin on RacV12 activation of HA-SGK1. HA-SGK1 was cotransfected with Rac1V12, and cell extracts were prepared from wortmanin or vehicle-treated cells as described in the legend to Fig. 1C. The lower insert depicts the amount of HA-SGK1 present in the assay. (D) The HGF-mediated activation of HA-SGK1 is not blocked by RacN17. MDCK cells were cotransfected with HA-SGK and an empty vector or a vector containing myc-tagged RacN17. The amount of SGK in the kinase assay is depicted in the HA-SGK blot. Verification of myc-RacN17 expression is shown in the lower blot. The kinase activity was carried out as described in the legend to Fig. 2A.

SGK1 wildtype (wt) and mutants were cloned into tagged mammalian expression vectors pCMV-Tag3B (myc tag) (Stratagene), pEGFP-C1 (GFP) (Clontech) and pKH3 (3X HA) (a gift from Ian Macara) using standard molecular cloning techniques. Active myc-tagged V12 Rac was a gift from A. Abo, Onyx (Richmond, CA). Myc-tagged N17 Rac (dead) and L63 Rho (active) were gifts from Alan Hall (University College London, UK). GFP-PH/AKT was a gift from Pfizer.

Cell culture, transfection and stimulation

MDCK cells were supplied by ATCC (Manassas, VA) and maintained in DMEM/F12 media with 15 mM HEPES and 10% FBS. Transfections were performed using LipofectAMINE 2000 Reagent according to the manufacturer's protocol (Life Technologies). Protein expression was confirmed by immunoblotting cell lysates with antibodies directed to the specific tag used (HA, HAF7; c-myc, 9E10 or A14) obtained from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Where indicated, cells were incubated with wortmanin (Calbiochem, La Jolla, CA) (50 nM) in fresh media for 1 hour prior to activation. For HGF stimulation, transfected cells were incubated overnight in serum-free media containing 0.2% BSA and stimulated in the presence or absence of wortmanin for 10 minutes with 50 ng/ml HGF (R&D Systems, Minneapolis, MN). For Rac1 experiments, active Rac1 (RacV12) or dominant-negative Rac1 (RacN17) was cotransfected with SGK1 and left overnight in media with 0.5% FBS prior to the kinase assay. For cAMP activation, transfected cells were incubated overnight in 0.5% FBS and the following morning treated with 1 mM 8Bromo-cAMP for 15 minutes before harvesting. For adhesion-mediated activation, cells were serum-starved overnight, trypsinized, washed twice and allowed to recover for 1 hour at 37°C in media with 0.2% BSA with or without wortmanin (50 nM) before plating on fibronectin-coated dishes for the times indicated. Cell extracts were prepared as previously described (Herrera, 1998; Shelly et al., 1998) and analyzed for protein expression and kinase activity.

Immunoblots and SGK1 kinase assay

Cell extracts were analyzed by western blot using anti-phospho-AKT (S473) and anti-AKT (Cell Signaling Technologies, Beverly, MA), anti-phospho-CREB (Upstate Biotechnology, Lake Placid, NY) antibodies. For kinase assays, cell extracts were immunoprecipitated with 2 μg of anti-HA (12CA5) antibody (Roche Molecular Chemicals, Indianapolis, IN) or 1 μg of GFP monoclonal antibody (Clontech) for 2 hours at 4°C. Immunoprecipitated SGK was washed in kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 2.5 mM BME, 0.1% Triton) before splitting into three aliquots. Kinase assays were performed with two of the aliquots as described by Park et al. (Park et al., 1999). Briefly, 40 μl of kinase buffer containing 100 μM ATP, 3 μCuries (γ-^{32}P)-ATP

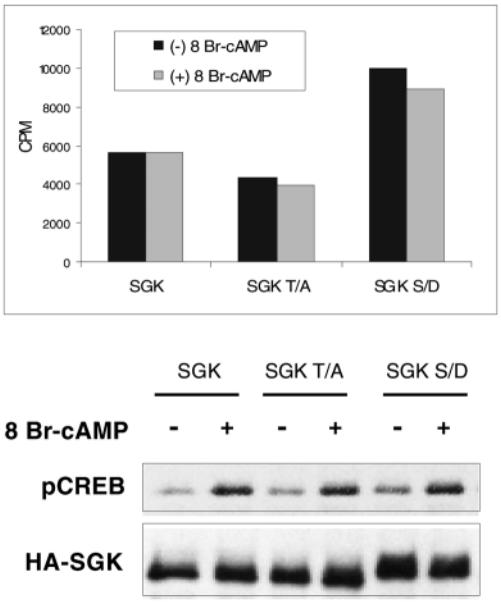

Fig. 3. SGK is not regulated by cAMP. Top, MDCK cells were transfected with wild-type, Thr369A mutant or S422D mutant SGK and cells were stimulated with 1 mM cAMP analog before SGK1 activity was measured. Kinase assay was carried out as described in the Materials and Methods. Bottom. The cell extracts were probed for phosphorylated CREB to verify cAMP stimulation and probed with anti-HA to determine the amount of HA-SGK in the kinase assay.

(3000-4000 cpm/pmol) (Amersham Pharmacia Biotech, Piscataway, NJ) and 10 μg of peptide substrate (KKRNRRLSVA) were added to the SGK beads and incubated at 30°C for 15 minutes. The reactions were stopped by adding 100 μl of 100 mM H_3PO_4, and then 35 μl was spotted onto phosphocellulose squares (Upstate Biotechnologies). The phosphocellulose squares were washed three times with 100 mM H_3PO_4, and the radioactivity was measured using a scintillation counter. The third aliquot was analyzed for SGK1 expression by immunoblotting using anti-HA (3F10) (Roche Molecular Chemicals). Immunoprecipitated GFP-SGK was blotted with anti-SGK (Upstate Biotechnology).

Immunofluorescence

MDCK cells were plated on two chamber slides (Lab-Tek, Nalgene NUNC International) and transfected with cDNA expression vectors encoding for GFP-SGK1, the PH-domain of Akt (Akt/PH-GFP) or pEGFP-C1 (empty vector) with or without vector encoding for Rac1 V12. After overnight incubation in 0.5% FBS, the media was replaced and cells were stimulated with HGF (50 ng/ml) for 10 minutes or as indicated in the legend to the figure. The cells were washed and fixed for 20 minutes in 4% formaldehyde then washed three times before mounting using Aqua Poly/Mount (Polysciences, Inc., Warrington, PA). Confocal Microscopy was performed on an Olympus Confocal microscope model IX70.

Adhesion-dependent modulation of FKHRL1 phosphorylation

MDCK cells transfected with either activated SGK1 (S/D) pKH3 or pKH3 (empty vector) were serum starved overnight. The following morning, the cells were trypsinized, washed and resuspended in media containing 0.2% BSA and 3 mM EGTA. Cells were kept in suspension for 1 hour at 37°C (t=1 hour) or lysed immediately (t=0). Total cell extracts were prepared as described in the kinase assay section and analyzed for the presence of phosphorylated FKHRL1 by western analysis. Anti-phospho-FKHRL1 (T32), (Cell Signaling Technologies) and anti-FKHRL1 (Upstate Biotechnologies) antibodies were used.

Results

Hepatocyte growth factor (HGF) activates SGK1 in MDCK cells

Treatment of epithelial cells, such as MDCK, with HGF leads to increased cell motility and biochemical changes that result in the scattering response (Stoker et al., 1987; Weidner et al., 1990; Weidner et al., 1993). Since the scatter response of the cells to HGF requires PI-3K activity (Khwaja et al., 1998; Potempa and Ridley, 1998), we have analyzed whether treatment of MDCK cells with HGF would lead to regulation of SGK activity in a manner similar to the activation of AKT by HGF (Coulonval et al., 2000; Liu, 1999; Xiao et al., 2001). We used HA-tagged SGK1 to transiently transfect MDCK cells and measured immunoprecipitated SGK1 activity in response to HGF treatment. In addition, we have analyzed the site-specific phosphorylation of endogenous AKT to compare its activation profile with the profile of SGK. As shown in Fig. 1A, stimulation of the cells with HGF (50 ng/ml) leads to a time-dependent activation of SGK1, reaching a maximum at 10 minutes. We compared the fold of activation of transfected SGK1 obtained by stimulation of MDCK cells with either that for HGF or IGF-1. Under our assay conditions, MDCK cells stimulated with HGF yielded activation of SGK1 to a similar extent as MDCK cells stimulated with IGF-1 (data not shown). The activation profile of HA-SGK1 in response to HGF parallels the phosphorylation of endogenous AKT at S473 (Fig. 1B). This phosphorylation has been previously shown to correlate with AKT activation (Vanhaesebroeck and Alessi, 2000). The HGF-induced activation of SGK1 or AKT is dependent on the activity of PI-3K since two unrelated chemical inhibitors of PI-3K activity, wortmanin and LY294002, prevent both AKT phosphorylation and SGK1 activation (Fig. 1C). (For LY294002 the data is not shown.) The activation of SGK1 by HGF was dependent on the integrity of the phosphorylation sites present at T256 and S422 of SGK1. Both single point mutants and the double site mutant SGK1 proteins failed to respond to HGF stimulation (data not shown). A similar behavior has been observed for IGF-1/H_2O_2 stimulation of SGK activity (Kobayashi and Cohen, 1999; Park et al., 1999). We studied the behavior of several mutant forms of SGK in response to various stimuli. The substitution S422D (S/D) residue has been shown to produce an activated form of SGK (Kobayashi and Cohen, 1999; Park et al., 1999). In our MDCK assay system, the S422D mutant produces a 2.4±0.4 (n=7) fold greater activation than wildtype. Although the SGK (S/D) has basal activity greater than wildtype, it still can be moderately stimulated by an agonist (Table 1).

Rac1-V12 activates HA-SGK1

The stimulation of MDCK cells with HGF induces activation of the Ras/Rac/Rho family of small GTP-binding proteins (Royal et al., 2000). We investigated whether cotransfection of activated forms of Rac1, RhoA or Rap1 would lead to HA-SGK1 activation. As shown in Fig. 2A, cotransfection of

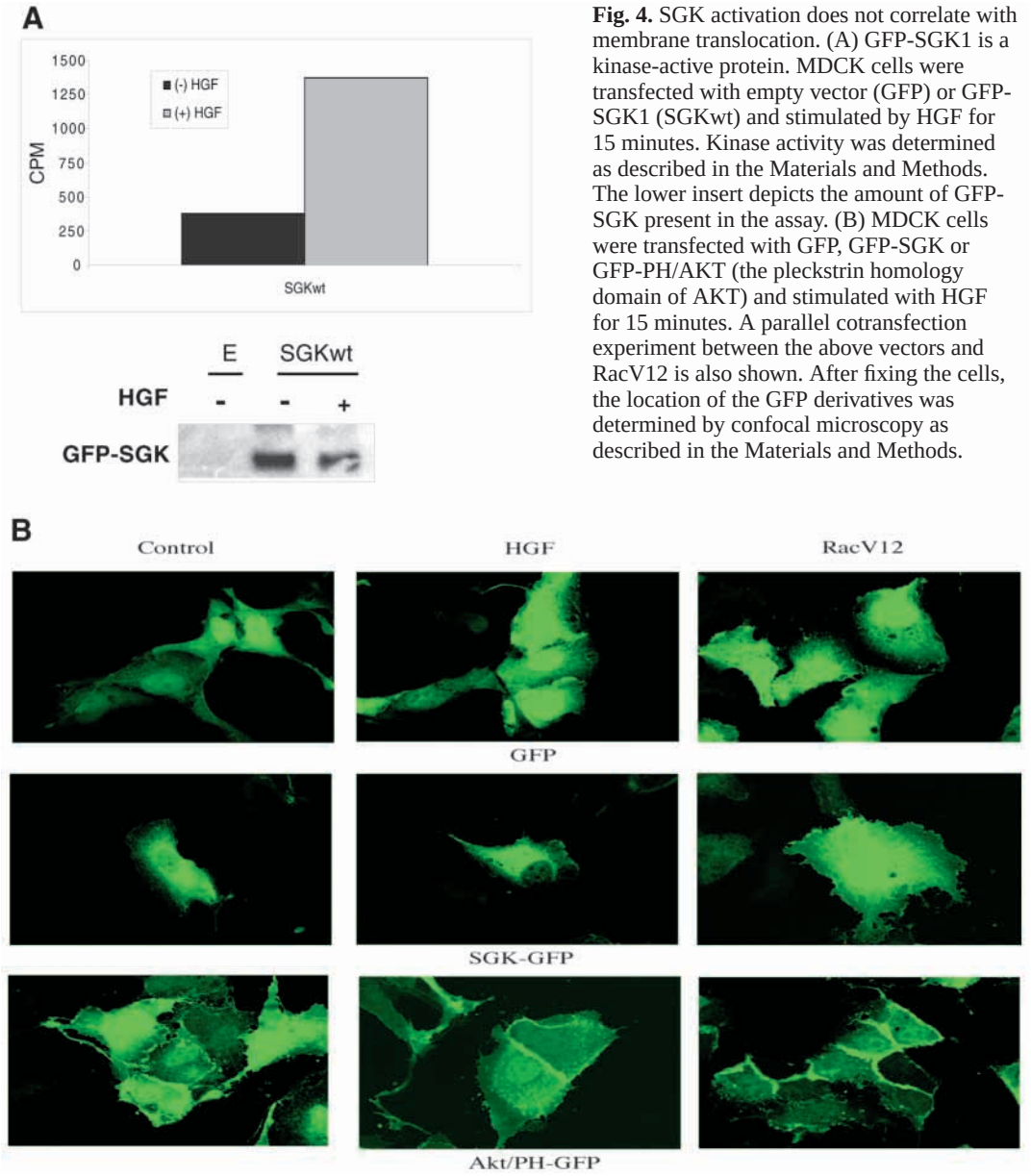

Fig. 4. SGK activation does not correlate with membrane translocation. (A) GFP-SGK1 is a kinase-active protein. MDCK cells were transfected with empty vector (GFP) or GFP-SGK1 (SGKwt) and stimulated by HGF for 15 minutes. Kinase activity was determined as described in the Materials and Methods. The lower insert depicts the amount of GFP-SGK present in the assay. (B) MDCK cells were transfected with GFP, GFP-SGK or GFP-PH/AKT (the pleckstrin homology domain of AKT) and stimulated with HGF for 15 minutes. A parallel cotransfection experiment between the above vectors and RacV12 is also shown. After fixing the cells, the location of the GFP derivatives was determined by confocal microscopy as described in the Materials and Methods.

activated Rac1 (Rac1V12) with HA-SGK1, but not with activated RhoA or Rap1, resulted in a highly reproducible activation of HA-SGK activity in a dose-dependent manner (Fig. 2B). Interestingly, the activation observed in response to Rac1-V12 was not blocked by wortmanin (Fig. 2C) although it still required an intact PDK1 (T256) or PDK2 (S422) phosphorylation site since activation was not observed in a SGK1 protein lacking either or both of these sites (data not shown). We next tested whether the Rac1 pathway was involved in mediating the HGF activation of SGK1. We compared the degree of HGF-induced activation of HA-SGK1 upon cotransfection with the dominant-negative form of Rac1 (RacN17) (Hall, 1998; Symons and Settleman, 2000). As shown in Fig. 2D, cotransfection of HA-SGK1 with RacN17 did not prevent the activation of HA-SGK by HGF. On the other hand, cotransfection of activated Rac1 with HA-SGK1 leads to increased activation upon stimulation with HGF as compared with the activity obtained in the presence of activated Rac1 alone or HGF stimulation in the absence of activated Rac1 (data not shown).

Analysis of the primary structure of SGK1 reveals the presence of other potential phosphorylation sites such as a tyrosine kinase site (Y124) and a PKA site (T369). We

Table 1. Degree of response to all stimuli

	Response	
Stimulus	SGK (wt)	**SGK (S/D)
HGF	2.2 (±0.2) *n*=9	1.5 (±0.1) *n*=3
Rac V12	2.2 (±0.2) *n*=10	1.6 (±0.7) *n*=2
Fibronectin	3.7 (±0.5) *n*=8	2.5 (±0.6) *n*=3

Table 1 represents the degree of response to all stimuli. SGK (S/D) has an intrinsic activity that is 2.4(±0.4) *n*=7 fold greater than SGK (wt) in the absence of stimuli. There was no activity detected in SGK mutants T256A, S422A or T256A/S422A using HGF, Rac1-V12 or fibronectin.

**SGK (SD) has the point mutation S422D.

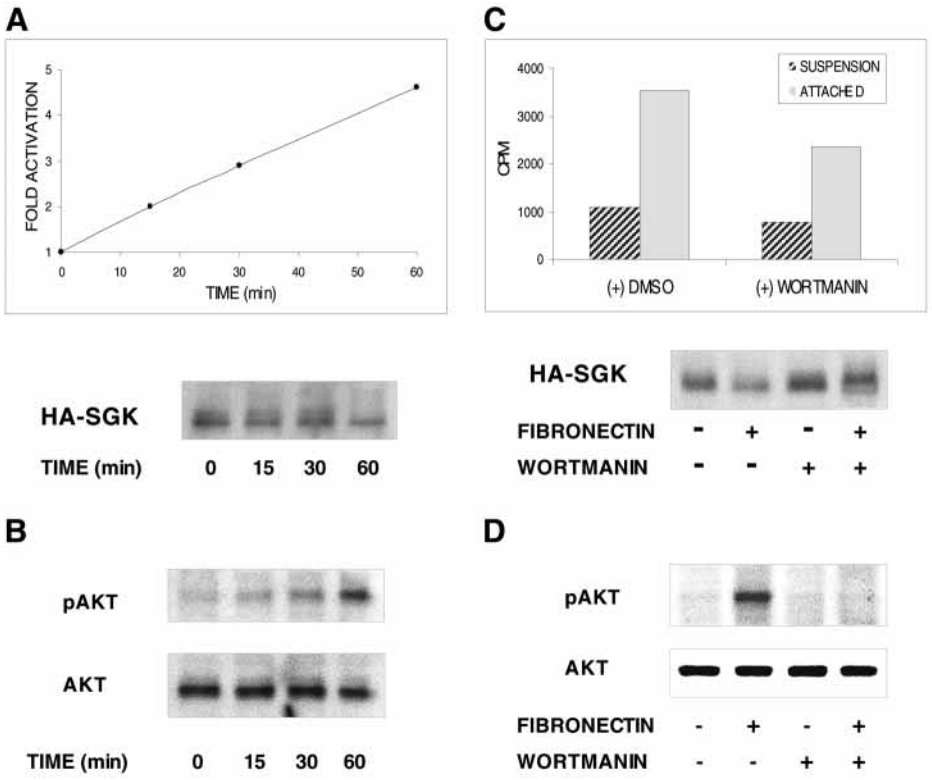

Fig. 5. Adhesion of MDCK cells to fibronectin activates HA-SGK1 in a wortmanin-insensitive manner. (A) Time course of HA-SGK1 activation upon cell adhesion to fibronectin-coated plates. Transfected MDCK cells were allowed to bind to fibronectin-coated plates, and HA-SGK1 activity was measured at the indicated times as described in the Materials and Methods. The insert depicts the level of HA-SGK1 present in the assay. (B) Adhesion-dependent activation of AKT. Cell extracts prepared as in (A) and were analyzed for the presence of phosphorylated AKT (pAKT). (C) Adhesion-mediated activation of HA-SGK1 is not blocked by wortmanin. HA-SGK1 activation by adhesion to fibronectin coated plates was studied in the presence or absence of wortmanin. After 30 minutes of adhesion to fibronectin, SGK1 activity was measured as in (A). The insert depicts the level of HA-SGK1 present in the assay. (D) Adhesion-dependent activation of AKT is blocked by wortmanin. Cells extracts were prepared as in (C) and were analyzed for the presence of phosphorylated AKT (T473) (pAKT).

analyzed whether activation of SGK1 results in tyrosine phosphorylation and whether the potential PKA site is a functional regulatory site. We failed to detect tyrosine phosphorylation of HA-SGK1 in response to HGF treatment. Similarly, we were unable to demonstrate a significant activation of HA-SGK1 by cAMP stimulation under conditions where we detect cAMP-mediated phosphorylation of the transcription factor CREB (Fig. 3). In addition, we mutated the potential PKA site and tested its role in the activation of HA-SGK1 in response to stimulation. Changing the potential PKA phosphorylation site to alanine produced an active protein that was still responsive to HGF or Rac1 V12. The level of activation of T369A mutant enzyme by Rac1 V12 or HGF was consistently only 75% of that the wild-type enzyme. While this work was being carried out, a report was published (Perrotti et al., 2001) describing cAMP regulation of SGK activity with results that are different from those that we describe above. The reason for these discrepancies is currently not clear.

We constructed a GFP-SGK1 fusion protein in order to study whether activation of SGK1 by HGF or RacV12 correlates with subcellular translocation. We compared GFP-SGK1 movement with that of the GFP-PH/AKT fusion protein, which is a marker for membrane recruitment (Raucher et al., 2000). As shown in Fig. 4A, the GFP-SGK1 fusion protein is expressed as a full-length, kinase-active protein in transfected MDCK cells. Treatment of GFP-SGK1- or GFP-PH/AKT-transfected cells with either HGF or cotransfection with RacV12 induces recruitment of the GFP-PH/AKT protein to the intercellular surfaces (Fig. 4B), indicating that activation of PI-3K has taken place. The GFP-SGK1 protein, by contrast, is not significantly recruited to the same structures, suggesting that HGF-and

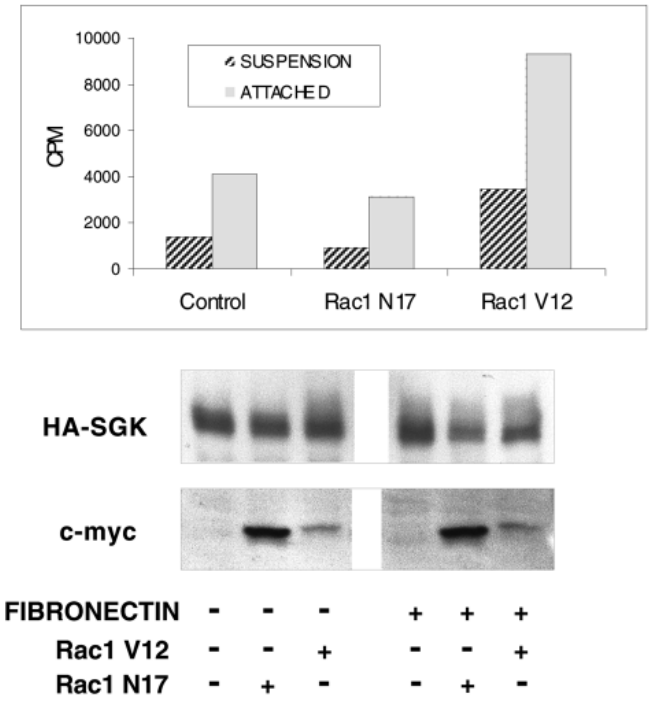

Fig. 6. Adhesion-mediated activation of HA-SGK1 is not blocked by Rac1N17. MDCK cells were cotransfected with HA-SGK1 and myc-Rac1V12 or myc-Rac1N17, allowed to adhere to fibronectin and the activation of HA-SGK1 was measured as described in Fig. 1. The lower insert depicts the levels of HA-SGK1, Rac1N17 and Rac1V12 present in the assay.

RacV12-mediated activation of SGK1 does not involve significant subcellular redistribution.

Adhesion-dependent activation of SGK1

It has previously been demonstrated that cell adhesion to the extracellular matrix via integrins induces activation of AKT (Banfic et al., 1998; Guilherme and Czech, 1998). Thus, we were interested in determining whether adhesion of MDCK cells to fibronectin would result in activation of transfected HA-SGK1. We measured and compared the kinase activity of SGK1 isolated from suspended and attached cells. We also followed the endogenous activation of AKT by monitoring its phosphorylation at S473.

Time course analysis of the activation of SGK1 upon interaction of MDCK cells with immobilized fibronectin showed that adhesion of the cells to the extracellular matrix induces a prolonged activation of SGK1 (Fig. 5A). The activation profile followed the spreading of the cells (data not shown). As previously reported (King et al., 1997), the adhesion of the cells also leads to AKT activation (Fig. 5B). However, it appears that the integrin-mediated activation of HA-SGK1 differs from AKT activation in that AKT phosphorylation was blocked by wortmanin (or LY294002). Although the basal activity of HA-SGK1 in the presence of wortmanin is reduced, the fold of activation was not (Fig. 5C,D). Similarly, LY294002 did not reduce the fold activation of SGK1 despite strong inhibition of AKT phosphorylation (data not shown).

Integrin-mediated activation of HA-SGK1 requires the intact phosphorylation sites that regulate HA-SGK1 activation in response to HGF or Rac1-V12. We therefore assessed the role of Rac1 in integrin-mediated activation of SGK1. Cotransfection of HA-SGK with dominant-negative Rac1 (RacN17) did not result in inhibition of adhesion-stimulated activation of SGK1 (Fig. 6). The RacN17 is acting as a true dominant negative in this system since it blocked the HGF-stimulated actin reorganization (data not shown). Cotransfection of HA-SGK1 with RacV12 results in increased activity in adherent cells compared with the effect of transfection of HA-SGK1 alone. The binding of cotransfected cells to fibronectin resulted in a greater activation than is obtained in the absence of RacV12, indicating that the adhesion-dependent activation is achieved by a parallel Rac-independent pathway (Fig. 6).

Activated SGK1 prevents de-attachment-induced dephosphorylation of FKHRL1

The activation of SGK1 by integrin or HGF may be connected to the modulation of cell survival. HGF activates an anti-apoptotic pathway (Bardelli et al., 1996) and blocks apoptosis of MDCK cells induced by loss of integrin-mediated cell attachment (anoikis) (Frisch and Francis, 1994), suggesting that both HGF and integrin signaling share a common pathway. We investigated whether the phosphorylation state of FKHRL1, a known modulator of cell survival (Liu et al., 2000), was regulated by cell attachment and whether this regulation could be influenced by SGK1. As shown in Fig. 7, deattachment of MDCK cells leads to a rapid loss of phosphorylated FKHRL1 (compare lanes 1-4 with lanes 7,8).

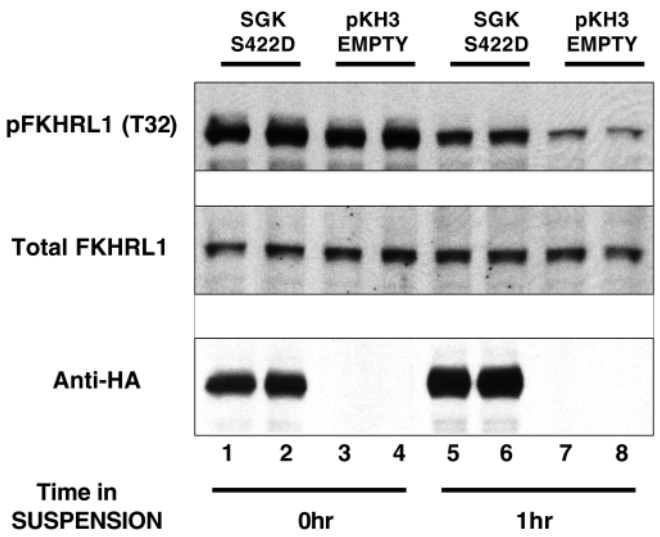

Fig. 7. Deattachment-induced FKHRL1 dephosphorylation is prevented by activated SGK1. MDCK cells were transfected with activated SGK1(S/D) (lanes 1, 2, 5 and 6) or empty vector (pKH3) (lanes 3, 4, 7 and 8) and serum starved overnight. Cells were deattached by trpysin incubation and kept in suspension for 1 hour (1 hour) or lysed immediately (0 hours). Duplicated cell extracts were prepared and analyzed for the presence of phosphorylated FKRHL1 (T32) (top panel), total FKRHL1 (middle panel) or for the presence of HA-SGK1 (bottom panel)

However, the presence of activated SGK1 prevented the dephosphorylation of FKHRL1 during the incubation period, suggesting that activated SGK1 provides survival signals similar to the one described for Akt (Brunet et al., 1999).

Discussion

SGK gene expression is under the control of growth factors and glucocorticoids (Webster et al., 1993a; Webster et al., 1993b). Northen blot analysis has shown that this kinase is expressed in several tissues, including the pancreas, skeletal muscle, liver, heart, placenta, kidney and brain (Waldegger et al., 1997). The expression of SGK has been correlated with aldosterone-mediated regulation of the epithelial sodium channel (Alvarez de la Rosa et al., 1999; Chen et al., 1999). In addition, the activity of the heterodimeric amino-acid transporter 4F2hc/LAT1 is associated with a non-selective cation channel that is regulated by SGK1 (Wagner et al., 2000). The expression of SGK1 is regulated by anisotonic and isotonic alterations of cell volume (Waldegger et al., 1997) as well as by hyperosmotic stress (Bell et al., 2000). It has been shown that the expression of SGK1 is deranged in diabetic nephropathy (Lang et al., 2000).

The role of SGK in signal transduction is poorly defined. However, as it belongs to the AGC class of kinases, its activity is regulated by PDK1, suggesting that it may participate in some of the pathways under the control of PI-3K (Currie et al., 1999; Czech, 2000; Kobayashi and Cohen, 1999; Park et al., 1999; Vanhaesebroeck and Alessi, 2000; Williams et al., 2000). Accordingly, it has been suggested that SGK participates in the regulation of GSK-3 (Kobayashi and Cohen, 1999) and that it functionally replaces the *Ypk1* gene in budding yeast

(Casamayor et al., 1999). On the other hand, it is also evident from the data presented in this paper that SGK1 activation can be accomplished by signaling pathways that are independent of PI-3K activation. While this paper was in preparation, a report describing activation of SGK by ERK5 independently of PDK1-mediated activation was published (Hayashi, 2001).

We analyzed the activation profiles of AKT and SGK upon stimulation with agonists or signaling intermediaries in order to compare the mechanism of activation of these two members of the AGC kinase family. As described in the Results section, we demonstrated that treatment of MDCK cells with HGF induces activation of both AKT and SGK in a PI-3K-dependent manner (Fig. 1A-C; Table 1). However, in contrast to HGF-mediated activation of SGK1, activation of SGK by RacV12 was shown to be wortmanin insensitive, suggesting that RacV12 activation does not require activation of PI-3K (Fig. 2A-C). It has been shown that Rac1-mediated activation of AKT in T cells requires PI-3K activity (Genot et al., 2000). It remains to be determined whether Rac1 activates AKT in MDCK cells and whether it is PI-3K independent as has been reported for the activation of AKT by cAMP (Filippa et al., 1999). We analyzed the possibility that cAMP also activates SGK, as treatment of granulosa cells with forskolin induces SGK phosphorylation (Gonzalez-Robayna et al., 2000). We observed the presence of a potential PKA site in SGK and mutated it to measure its contribution to SGK activation. Unlike researchers in previous reports (Perrotti et al., 2001), we failed to demonstrate cAMP-mediated activation of SGK under conditions that lead to phosphorylation of CREB (Fig. 3).

Our analysis of adhesion-dependent activation of SGK revealed that adhesion of MDCK cells to fibronectin activates SGK in a wortmanin-independent manner. This is in contrast to observations for AKT activation under the same conditions (Fig. 5A-C). Integrin-mediated activation of SGK was not inhibited by the neutralization of Rac activation. Indeed, we saw an additive activation mediated by RacV12 and fibronectin adhesion, indicating that there are other pathways that lead to SGK activation. Significantly, the activation of SGK is still under the structural requirements that regulate PDK1 activation of SGK, since mutations in the PDK1 or PDK2 sites produce an inactive SGK protein. There are conditions, however, in which PDK1 activity does not require input from the PI-3K pathway (Vanhaesebroeck and Alessi, 2000), thus explaining the need for the PDK1 site without requiring activation of PI-3K.

The regulation of SGK activity in MDCK cells by both HGF and cell adhesion suggests that SGK may participate in signaling pathways that modulate cell motility. However, recently published information (Brunet et al., 2001; Liu et al., 2000) points to a functional role for SGK that is complementary to the role that AKT plays in promoting cell survival by directly phosphorylating and inactivating the pro-apoptotic proteins FKHR and BAD (Brunet et al., 1999). Here we have shown that SGK1 also mediates signaling associated with cell survival. The activation of SGK1 by HGF or integrin may connect this protein to the protection of MDCK from anoikis since the deattachment-induced dephosphorylation of FKHRL1 is significantly reduced by expression of activated SGK1 (Fig. 7).

In conclusion, we present experimental evidence that SGK1 activation can be accomplished via HGF, PI-3K-dependent pathways and by integrin-mediated, PI-3K-independent pathways. In addition, activation of SGK1 by the small GTP-binding protein Rac1 has been observed. These results suggest that activation of SGK1 could be achieved through signaling pathways involved in the regulation of cell survival, cell-cell and cell-matrix interactions.

References

Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N. and Avruch, J. (1998). 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. *Curr. Biol.* **8**, 69-81.

Alliston, T. N., Maiyar, A. C., Buse, P., Firestone, G. L. and Richards, J. S. (1997). Follicle stimulating hormone-regulated expression of serum/glucocorticoid-inducible kinase in rat ovarian granulosa cells: a functional role for the Sp1 family in promoter activity. *Mol. Endocrinol.* **11**, 1934-1949.

Alvarez de la Rosa, D., Zhang, P., Naray-Fejes-Toth, A., Fejes-Toth, G. and Canessa, C. M. (1999). The serum and glucocorticoid kinase sgk increases the abundance of epithelial sodium channels in the plasma membrane of *Xenopus* oocytes. *J. Biol. Chem.* **274**, 37834-37839.

Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P. and Alessi, D. R. (1999). PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr. Biol.* **9**, 393-404.

Banfic, H., Downes, C. P. and Rittenhouse, S. E. (1998). Biphasic activation of PKBalpha/Akt in platelets. Evidence for stimulation both by phosphatidylinositol 3,4-bisphosphate, produced via a novel pathway, and by phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **273**, 11630-11637.

Bardelli, A., Longati, P., Albero, D., Goruppi, S., Schneider, C., Ponzetto, C. and Comoglio, P. M. (1996). HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. *EMBO J.* **15**, 6205-6212.

Bell, L. M., Leong, M. L., Kim, B., Wang, E., Park, J., Hemmings, B. A. and Firestone, G. L. (2000). Hyperosmotic stress stimulates promoter activity and regulates cellular utilization of the serum- and glucocorticoid-inducible protein kinase (Sgk) by a p38 MAPK-dependent pathway. *J. Biol. Chem.* **275**, 25262-25272.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J. and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-868.

Brunet, A., Park, J., Tran, H., Hu, L. S., Hemmings, B. A. and Greenberg, M. E. (2001). Protein kinase SGK mediates survival signals by phosphorylating the Forkhead transcription factor FKHRL1 (FOXO3a). *Mol. Cell. Biol.* **21**, 952-965.

Buse, P., Tran, S. H., Luther, E., Phu, P. T., Aponte, G. W. and Firestone, G. L. (1999). Cell cycle and hormonal control of nuclear-cytoplasmic localization of the serum- and glucocorticoid-inducible protein kinase, Sgk, in mammary tumor cells. A novel convergence point of anti-proliferative and proliferative cell signaling pathways. *J. Biol. Chem.* **274**, 7253-7263.

Casamayor, A., Torrance, P. D., Kobayashi, T., Thorner, J. and Alessi, D. R. (1999). Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. *Curr. Biol.* **9**, 186-197.

Chen, S. Y., Bhargava, A., Mastroberardino, L., Meijer, O. C., Wang, J., Buse, P., Firestone, G. L., Verrey, F. and Pearce, D. (1999). Epithelial sodium channel regulated by aldosterone-induced protein sgk. *Proc. Natl. Acad. Sci. USA* **96**, 2514-2519.

Cheng, X., Ma, Y., Moore, M., Hemmings, B. A. and Taylor, S. S. (1998). Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **95**, 9849-9854.

Coulonval, K., Vandeput, F., Stein, R. C., Kozma, S. C., Lamy, F. and Dumont, J. E. (2000). Phosphatidylinositol 3-kinase, protein kinase B and ribosomal S6 kinases in the stimulation of thyroid epithelial cell proliferation by cAMP and growth factors in the presence of insulin. *Biochem. J.* **348**, 351-358.

Currie, R. A., Walker, K. S., Gray, A., Deak, M., Casamayor, A., Downes, C. P., Cohen, P., Alessi, D. R. and Lucocq, J. (1999). Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and

localization of 3-phosphoinositide-dependent protein kinase-1. *Biochem. J.* **337**, 575-583.

Czech, M. P. (2000). PIP2 and PIP3: complex roles at the cell surface. *Cell* **100**, 603-606.

Delcommenne, M., Tan, C., Gray, V., Rue, L., Woodgett, J. and Dedhar, S. (1998). Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc. Natl. Acad. Sci. USA* **95**, 11211-11216.

Filippa, N., Sable, C. L., Filloux, C., Hemmings, B. and Van Obberghen, E. (1999). Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase. *Mol. Cell Biol.* **19**, 4989-5000.

Frisch, S. M. and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124**, 619-626.

Genot, E. M., Arrieumerlou, C., Ku, G., Burgering, B. M., Weiss, A. and Kramer, I. M. (2000). The T-cell receptor regulates Akt (protein kinase B) via a pathway involving Rac1 and phosphatidylinositide 3-kinase. *Mol. Cell Biol.* **20**, 5469-5478.

Gonzalez-Robayna, I. J., Falender, A. E., Ochsner, S., Firestone, G. L. and Richards, J. S. (2000). Follicle-Stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-lnduced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. *Mol. Endocrinol.* **14**, 1283-1300.

Guilherme, A. and Czech, M. P. (1998). Stimulation of IRS-1-associated phosphatidylinositol 3-kinase and Akt/protein kinase B but not glucose transport by beta1-integrin signaling in rat adipocytes. *J. Biol. Chem.* **273**, 33119-33122.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.

Herrera, R. (1998). Modulation of hepatocyte growth factor-induced scattering of HT29 colon carcinoma cells. Involvement of the MAPK pathway. *J. Cell Sci.* **111**, 1039-1049.

Khwaja, A., Lehmann, K., Marte, B. M. and Downward, J. (1998). Phosphoinositide 3-kinase induces scattering and tubulogenesis in epithelial cells through a novel pathway. *J. Biol. Chem.* **273**, 18793-18801.

King, W. G., Mattaliano, M. D., Chan, T. O., Tsichlis, P. N. and Brugge, J. S. (1997). Phosphatidylinositol 3-kinase is required for integrin-stimulated AKT and Raf-1/mitogen-activated protein kinase pathway activation. *Mol. Cell. Biol.* **17**, 4406-4418.

Kobayashi, T. and Cohen, P. (1999). 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. *Biochem. J.* **339**, 319-328.

Kobayashi, T., Deak, M., Morrice, N. and Cohen, P. (1999). Characterization of the structure and regulation of two novel isoforms of serum- and glucocorticoid-induced protein kinase. *Biochem. J.* **344**, 189-197.

Lang, F., Klingel, K., Wagner, C. A., Stegen, C., Warntges, S., Friedrich, B., Lanzendorfer, M., Melzig, J., Moschen, I., Steuer, S. et al. (2000). Deranged transcriptional regulation of cell-volume-sensitive kinase hSGK in diabetic nephropathy. *Proc. Natl. Acad. Sci. USA* **97**, 8157-8162.

Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. and Parker, P. J. (1998). Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* **281**, 2042-2045.

Liu, D., Yang, X. and Songyang, Z. (2000). Identification of CISK, a new member of the SGK kinase family that promotes IL-3-dependent survival. *Curr. Biol.* **10**, 1233-1236.

Liu, Y. (1999). Hepatocyte growth factor promotes renal epithelial cell survival by dual mechanisms. *Am. J. Physiol.* **277**, F624-F633.

Lynch, D. K., Ellis, C. A., Edwards, P. A. and Hiles, I. D. (1999). Integrin-linked kinase regulates phosphorylation of serine 473 of protein kinase B by an indirect mechanism. *Oncogene* **18**, 8024-8032.

Maiyar, A. C., Huang, A. J., Phu, P. T., Cha, H. H. and Firestone, G. L. (1996). p53 stimulates promoter activity of the sgk. Serum/glucocorticoid-inducible serine/threonine protein kinase gene in rodent mammary epithelial cells. *J. Biol. Chem.* **271**, 12414-12422.

Hayashi, M., Tapping, R. I., Chao, T. H., Lo, J.-F., King, C. C., Yang, Y. and Lee, J. D. (2001). BMK1 mediates growth factor-induced cell proliferation through direct cellular activation of SGK. *J. Biol. Chem.* **276**, 8631-8634.

Park, J., Leong, M. L., Buse, P., Maiyar, A. C., Firestone, G. L. and Hemmings, B. A. (1999). Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. *EMBO J.* **18**, 3024-3033.

Perrotti, N., He, R. A., Phillips, S. A., Renfrew Haft, C. and Taylor, S. I. (2001). Activation of serum- and glucocorticoid-induced protein kinase (SGK) by cyclic AMP and insulin. *J. Biol. Chem.* **276**, 9406-9412.

Potempa, S. and Ridley, A. J. (1998). Activation of both MAP kinase and phosphatidylinositide 3-kinase by Ras is required for hepatocyte growth factor/scatter factor-induced adherens junction disassembly. *Mol. Biol. Cell* **9**, 2185-2200.

Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A. and Thomas, G. (1998). Phosphorylation and activation of p70s6k by PDK1. *Science* **279**, 707-710.

Raucher, D., Stauffer, T., Chen, W., Shen, K., Guo, S., York, J. D., Sheetz, M. P. and Meyer, T. (2000). Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. *Cell* **100**, 221-228.

Royal, I., Lamarche-Vane, N., Lamorte, L., Kaibuchi, K. and Park, M. (2000). Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. *Mol. Biol. Cell* **11**, 1709-1725.

Shelly, C., Petruzzelli, L. and Herrera, R. (1998). PMA-induced phenotypic changes in K562 cells: MAPK-dependent and -independent events. *Leukemia* **12**, 1951-1961.

Stoker, M., Gherardi, E., Perryman, M. and Gray, J. (1987). Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* **327**, 239-242.

Symons, M. and Settleman, J. (2000). Rho family GTPases: more than simple switches. *Trends Cell Biol.* **10**, 415-419.

Toker, A. and Newton, A. C. (2000). Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J. Biol. Chem.* **275**, 8271-8274.

Vanhaesebroeck, B. and Alessi, D. R. (2000). The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* **346**, 561-576.

Wagner, C. A., Broer, A., Albers, A., Gamper, N., Lang, F. and Broer, S. (2000). The heterodimeric amino acid transporter 4F2hc/LAT1 is associated in *Xenopus* oocytes with a non-selective cation channel that is regulated by the serine/threonine kinase sgk-1. *J. Physiol. (Lond.)* **526**, 35-46.

Waldegger, S., Barth, P., Raber, G. and Lang, F. (1997). Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during anisotonic and isotonic alterations of cell volume. *Proc. Natl. Acad. Sci. USA* **94**, 4440-4445.

Webster, M. K., Goya, L. and Firestone, G. L. (1993a). Immediate-early transcriptional regulation and rapid mRNA turnover of a putative serine/threonine protein kinase. *J. Biol. Chem.* **268**, 11482-11485.

Webster, M. K., Goya, L., Ge, Y., Maiyar, A. C. and Firestone, G. L. (1993b). Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Mol. Cell Biol.* **13**, 2031-2040.

Weidner, K. M., Behrens, J., Vandekerckhove, J. and Birchmeier, W. (1990). Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. *J. Cell Biol.* **111**, 2097-2108.

Weidner, K. M., Hartmann, G., Sachs, M. and Birchmeier, W. (1993). Properties and functions of scatter factor/hepatocyte growth factor and its receptor c-Met. *Am. J. Respir. Cell. Mol. Biol.* **8**, 229-237.

Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. and Alessi, D. R. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in a ctivating AGC kinases defined in embryonic stem cells. *Curr. Biol.* **10**, 439-448.

Xiao, G. H., Jeffers, M., Bellacosa, A., Mitsuuchi, Y., Vande Woude, G. F. and Testa, J. R. (2001). Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. *Proc. Natl. Acad. Sci. USA* **98**, 247-252.