Serum and glucocorticoid-inducible kinase SGK phosphorylates and negatively regulates B-Raf

Bao-Hong Zhang¹, Eric Tang¹, Tianqing Zhu¹, Michael Greenberg³, Anne Vojtek¹, and Kun-Liang Guan¹,²*

¹Department of Biological Chemistry and ²Institute of Gerontology, University of Michigan Ann Arbor, MI, USA 48109-0606.

³Children's Hospital and Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115

*To whom correspondence should be addressed
734-763-3030 (Tel); 734-763-4581 (Fax); kunliang@umich.edu (E-mail)

Running title: Inhibition of B-Raf by SGK
Summary

Phosphorylation can both positively and negatively regulate activity of the Raf kinases. AKT has been shown to phosphorylate and inhibit C-Raf activity. We have recently reported that Akt negatively regulates B-Raf kinase activation by phosphorylating multiple residues within its amino-terminal regulatory domain. Here we investigate the regulation of B-Raf by serum and glucocorticoid-inducible kinase, SGK, which shares close sequence identity with the catalytic domain of Akt, but lacks the pleckstrin homology domain. We observed that SGK inhibits B-Raf activity. Comparison of substrate specificity between SGK and Akt indicates that SGK is a potent negative regulator of B-Raf. In contrast to Akt, SGK negatively regulates B-Raf kinase activity by phosphorylating only a single Akt consensus site Ser364. Under similar experimental conditions, SGK displays a measurable stronger inhibitory effect on B-Raf kinase activity than Akt, whereas Akt exhibits more inhibitory effect on the forkhead transcription factor FKHR. The selective substrate specificity is correlated with an enhanced association between Akt or SGK and their preferred substrates FKHR and B-Raf, respectively. These results indicate that B-Raf kinase activity is negatively regulated by Akt and SGK, and suggest that the cross-talk between the B-Raf and other signaling pathways can be mediated by both Akt and SGK.

Keywords: B-Raf/kinase/phosphorylation/Ras/Akt/SGK
Introduction

Diverse extracellular stimuli activate the Ras-mitogen activated protein (MAP) kinase pathway, also known as extracellular signal regulated kinase (ERK). The Raf protein kinase plays an essential role in transmitting signals from Ras-GTP to activation of MEK (also known as MAP kinase kinase) and MAP kinase. Activation of the Ras-MAP kinase pathway has been implicated in modulation of a wide variety of cellular responses, including cell proliferation, differentiation, cell death, and development (1-4). The Ras-MAP kinase pathway is, therefore, subject to tight regulation in response to a combination of extracellular stimuli. Cross talk between different signaling pathways is likely to be a critical mechanism for the proper regulation of this multi-functional pathway (5,6).

All MAP kinase modules consist of three kinases acting in a sequence and are conserved during evolution. The regulation of Raf-MEK-ERK cascade has been extensively studied and is one of the best-understood MAP kinase cascades (7,8). MEK directly phosphorylates ERK on a conserved TXY motif between the kinase subdomain VII and VIII and activates ERK (9). Similarly, Raf directly phosphorylates MEK on two serine residues between the kinase subdomain of VII and VIII and activates MEK (10-12). However, the regulation of Raf is much more complex and not fully understood.

Three isoforms of Raf proteins have been found in the human genome: A-Raf, B-Raf, and C-Raf (also known as Raf-1) (13). Both B-Raf and C-Raf have been implicated in phosphorylating and activating MEK while the function of A-Raf is less documented. Structurally, Raf kinases contain three conserved regions, CR1, CR2, and CR3 (14). The CR1 region consists of a Ras binding domain (RBD) and a cysteine rich domain, both of which bind Ras and are important for Raf activation by Ras(15-18). The CR2 region is rich in serine and threonine residues and multiple phosphorylation sites within this region are responsible for Raf kinase activation (4). The CR3 region is the kinase catalytic domain and phosphorylation of residues within this region also responsible for Raf activation (4). Deletion of the N-terminal regulatory domains of Raf results in activation of Raf kinase
activity, suggesting that the N-terminal domain of Raf inhibits its kinase activity (19). In fact, viral oncogenic Raf was originally isolated as an oncogene, which lacks the N-terminal regulatory domain (20).

Raf activation by Ras is a feature conserved in C. elegans, Drosophila, and mammals. The interaction between Raf and active Ras is common for all Raf family kinases, and although Ras-Raf binding is necessary, it is not sufficient for Raf activation (21-24). Studies performed with C-Raf have demonstrated that phosphorylation of Ser338 and Tyr341 are essential for Raf activation by Ras, growth factor, and PMA (25-27). Using phospho-specific antibodies, Mason et al. have shown that Ras induces predominantly Ser338 phosphorylation, whereas Src mainly stimulates Tyr341 phosphorylation (27). Synergistic activation of Raf was observed when both sites were phosphorylated. Recently, we have demonstrated that phosphorylation of Thr598 and Ser601 in B-Raf, which correspond to Thr491 and Ser494 of C-Raf, are essential for B-Raf activation (28). Phosphorylation of these two sites is stimulated by active Ras. Simultaneous substitutions of Thr598 and Ser601 by acidic residues result in a significant elevation of B-Raf activity. Interestingly, Thr598 and Ser601 of B-Raf are conserved in all Raf family kinases and are likely to be common phosphorylation sites for Raf activation (Chong and Guan, unpublished data).

Raf kinase activity is also negatively regulated by phosphorylation. PKA phosphorylates Ser43 of C-Raf and inhibits its activity (29-32). Recently, Akt has been reported to phosphorylate Ser259, which also inhibits C-Raf kinase activity (33). We have shown that B-Raf kinase activity is inhibited by Akt through multiple sites within the CR2 region (34).

Activation of the phosphatidylinositol 3-kinase (PI3K) leads to an increase in production of phosphoinositol phosphate second messages (35). These lipid messages regulate activity and localization of a number of target proteins, including those containing pleckstrin homology (PH) domains. Akt, a Ser/Thr kinase also known as protein kinase B (PKB), was identified as a viral transforming oncogene and plays an important role in
promoting cell survival (36). Akt contains a PH domain and is a major downstream target of PI3K. Akt is phosphorylated and activated by phospholipid dependent kinase (PDK) (37,38), which is regulated by the phosphoinositide phosphate lipid messages. Several Akt targets have been identified, including GSK3 and p70S6 kinase, transcription factor FKHR, proteins associated with apoptosis, and Raf kinase (36). The serum and glucocorticoid inducible kinase (SGK), a novel member of the serine/threonine protein kinase gene family, shares significant sequence identity with Akt (39). Several groups have reported that SGK could also be activated by the PI3K pathway (40,41). PDK1 is likely to directly phosphorylate and enhance SGK activity (41,42). However, physiological targets of SGK are largely unknown. Recently, Brunet et al reported that SGK could phosphorylate and inhibit the FKHRL1 transcription factor (43). Interestingly, SGK and Akt selectively phosphorylate different residues in FKHRL1, demonstrating that Akt and SGK coordinately regulate the function of FKHRL1 by phosphorylating this transcription factor on distinct sites.

We investigated the role of SGK in B-Raf regulation. Our results demonstrated that SGK is potent to phosphorylate and inactivate B-Raf. A preferential association of SGK and B-Raf supports the effect of SGK on inhibition of B-Raf. SGK inhibits B-Raf mainly through phosphorylation of S364. Under similar experimental conditions, SGK displayed a slight higher potency to inhibit B-Raf. In contrast, Akt appears more effective in phosphorylating and inhibiting the FKHR transcription factor than SGK. This report demonstrates that SGK plays an important role in the negative regulation of Raf.
EXPERIMENTAL PROCURES

**DNA Constructs and Recombinant Proteins**—Full length human B-Raf cDNA which was subcloned into hemagglutinin antigen (HA)-tagged mammalian expression vector pcDNA3 and the constitutive active B-Raf mutant (HA-B-RafED in which Thr598 and S601 were substituted by acidic residues glutamic acid and aspartic acid, respectively), have been described previously (28). GST-B-Raf and other B-Raf mutants which contain Akt consensus site(s), including HA-B-RafA (Ser363 was mutated to alanine), HA-B-RafAA (both Ser428 and Thr439 were replaced by alanine residues) and HA-B-RafAAA (Ser364, Ser428 and Thr439 were all mutated to alanines) were described previously (34). Wild-type SGK (HA-SGK), constitutive active SGK (HA-SGK-S422D) and kinase dead SGK (HA-SGK-K127M) were generously provided by Dr. Stuart Decker (PFIZER INC, Ann Arbor) (42). Other DNA constructs including constitutively active Akt (HA-Akt-myrr), mammalian cell expressed FKHR (Flag-FKHR) and its constitutive active mutant (Flag-FKHRAAA in which three Akt phosphorylation sites, Thr24, Ser256 and Ser319, were all substituted with alanine residues) were reported as previously (44). Amino terminal (FKHR-N) and carboxyl-terminal (FKHR-C) fragments of FKHR which encompasses residues 1-257 and 211-416, respectively, were cloned into bacteria expression vector PGEX-KG (44). The expression and purification of glutathione S-transferase (GST) fusion proteins were performed using DH5α as described previously (34).

**Kinase Assays**—Lysates for immunoprecipitation experiments were prepared from transfected HEK293 cells grown on 6-well plates or 10 cm tissue culture dishes in DMEM medium supplemented with 10% fetal bovine serum. Briefly, cells were washed twice with cold PBS and lysed on ice in 1 ml lysis buffer (25 mM HEPES pH7.5, 150 mM NaCl, 1% Triton-X100, 0.1% SDS, 0.5 mM EDTA, 0.025% mercaptoethanol, 1 mM NaF, 200 μM Na3VO4, 200 μM phenylmethysulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin). The lysates were cleared by centrifugation at 14,000Xg for 10 minutes at 4°C to remove insoluble debris. Immunoprecipitation was performed by incubation with
appropriate antibodies for 1.5hrs at 4°C followed by incubation for further 1hr at 4°C with Protein G-Sepharose. Immunoprecipitates were washed three times with lysis buffer and once with PBS. B-Raf kinase activity was measured by coupled assay using GST-MEK, GST-ERK and GST-Elk as sequential substrates, as described previously (28). ERK kinase activity was determined using GST-Elk as a substrate (28).

For the Akt/SGK kinase assays, HA-SGK or HA-Akt immunoprecipitates were incubated for 30 min at 30°C with 40 µl reaction mixture containing 5 mM synthetic peptide (KKRNRRRLSVA) as a substrate, 18 mM Hepes, 10 mM MgCl2, 1 mM DTT, 50 mM ATP, 1 µM PKA inhibitor peptide (Calbiochem, CA, USA) and 10 μCi [γ-32P]ATP. 20 µl of 40% TCA was added to the supernatant and incubated for 5 min at room temperature. 40 µl of above fraction was spotted onto P81 Whatmann paper. The paper was washed extensively with 80 mM phosphoric acid, once with ethanol and counted.

**Reporter Assay**-293 cells in 12 well plates were co-transfected with 200 ng Flag-FKHR or Flag-FKHRAAA, 100 ng luciferase reporter (3XIRS-luc) and pCMV-βGal. Luciferase assays were performed as described previously (44).

**Coimmunoprecipitation Assays**-For examination of interaction between B-Raf and SGK or Akt, GST-vector or GST-B-Raf was cotransfected with HA-SGK or HA-Akt in HEK293 cells grown in 10 cm plates. 48h after transfection, cells were washed twice with ice-cold PBS and lysed in 10 mM HEPES pH7.5, 50 mM NaCl, 1% Triton-X100, 2 mM EDTA, 0.1% mercaptoethanol, 50 mM NaF, 1 mM Na3VO4, 200 µM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin. Glutathione-agarose beads were added to the cell lysates to purify GST-B-Raf. GST-B-Raf was then eluted in 10 mM glutathione in 50 mM tris, pH8.0 The glutathione-eluted samples were
subjected to SDS-PAGE and analyzed by Western blot with anti-GST and anti-HA antibodies. Similar experiments were performed for association of FKHR and SGK or Akt with cells cotransfected with Flag-FKHR and HA-SGK or HA-Akt, except Flag-FKHR was immunoprecipitated with anti-Flag M2 antibody. To immunoprecipitate endogenous SGK, two 10 cm HEK293 cells were immunoprecipitated using antiserum specific for SGK (43). The immunoprecipitated samples were probed with anti-B-Raf antibody.
Results

Inhibition of B-Raf by SGK-We have previously shown that B-Raf can be phosphorylated and inhibited by Akt. To test whether SGK plays a role in B-Raf regulation, we examined B-Raf kinase activity in the presence or absence of SGK. HA-B-Raf was transfected in HEK293 cells with SGK-S422D which is a constitutively active SGK mutant containing the activation phosphorylation site Ser422 substituted by an aspartic residue (42). HA-SGK-S422D was used because wild type SGK has low activity in the absence of serum stimulation. We observed that HA-SGK-S422D decreased the basal kinase activity of B-Raf, which was assayed by an in vitro coupled MEK kinase assay (Fig. 1, lanes 2 and 3). The effect of SGK on Ras-induced B-Raf activity was also examined. The RasV12 induced B-Raf activity was also inhibited by HA-SGK-S422D (64% inhibition, Fig.1, lanes 4 and 5). However, under similar conditions, the HA-Akt-myr, a constitutively active Akt containing a myristylation signal, also inhibited B-Raf (50% inhibition, Fig. 1, lane 7). As a negative control, the kinase inactive mutant of HA-SGK-K127M mutant was tested and showed no effect on RasV12 induced B-Raf activation (Fig.1, lane 6). These results demonstrated that kinase activity of SGK is essential for its ability to inhibit B-Raf activity.

SGK kinase activity is known to be stimulated by serum (40,41). Similarly, B-Raf kinase activity is stimulated by serum. We tested whether SGK can inhibit the serum induced B-Raf activation. Wildtype SGK, but not the kinase inactive SGK, effectively blocked B-Raf activation (Fig.1, lanes 8-11). Under identical conditions, Akt-myr is less effective. Our data indicate that SGK is a negative regulator of Raf.

Inhibition of B-Raf by SGK requires the presence of Ser364 in B-Raf-Biochemical characterizations demonstrate that SGK recognize a consensus phosphorylation site (RXRXXS/T) similar to that of Akt (40,41). B-Raf contains three putative Akt/SGK phosphorylation sites. Our previous studies indicated that Ser364 and Ser428 are phosphorylation sites by Akt (34). We examined whether SGK also uses similar sites to
inhibit B-Raf activity. HA-B-Raf-A, which has the Ser364 substituted by alanine residue, is resistant to inhibition by HA-SGK-422D (Fig. 2A, lanes 7 and 8), indicating that Ser364 in B-Raf is a target site for SGK. In contrast, HA-B-Raf-AA, which has both Ser428 and Thr439 substituted by alanine residues, still can be inhibited by HA-SGK-S422D (Fig. 2A, lanes 9 and 10). These results suggest that Ser428 and Thr439 in B-Raf are not required for its inhibition by SGK. As predicted, HA-B-Raf-AAA, which has all three Akt consensus sites replaced by alanine residues, is also not inhibited by HA-SGK-S422D (Fig.1, lanes 11 and 12). In the same experiments, the basal activity of wildtype B-Raf is effectively inhibited by HA-SGK-S422D (Fig. 2A, lane 1 and 2). Similarly, activation of wildtype B-Raf by serum is also inhibited by SGK (Fig. 2A, lanes 3 and 4). These results demonstrate that Ser364 in B-Raf is essential for inhibition by SGK.

We wanted to test whether B-Raf-induced ERK activation is inhibited by SGK in transfected HEK293 cells. Myc-ERK is co-transfected with HA-B-Raf in the presence or absence of SGK. Kinase activity of immunoprecipitated ERK was determined using Elk-1 as a substrate. Wild type B-Raf co-transfection can induce a low level of ERK activation which is inhibited by HA-SGK-S422D (Fig. 2B, lanes 1-3). We have previously identified important activation phosphorylation sites in B-Raf (28). Substitution of these sites by acidic residues created a constitutively active HA-B-Raf-ED. ERK activation induced by B-Raf-ED was also suppressed by SGK while kinase inactive HA-SGK-K127M had no inhibition at all (Fig. 2B, lanes 4-6). As a comparison, Akt-myr displayed a moderate inhibition of ERK activation by HA-B-Raf-ED mutant (Fig. 2B, lane 7). To further confirm the requirement of Ser364 in B-Raf inhibition by SGK, mutants of HA- tagged B-Raf-A, B-Raf-AA, and B-Raf-AAA were examined. These alanine mutations were constructed under the wild type B-Raf background and resulted in an elevation of basal kinase activity (Fig. 2B). SGK inhibited HA-B-Raf-AA but not HA-B-Raf-A or HA-B-Raf-AAA-induced Myc-ERK activation (Fig. 2B, lanes 8-13). These results are completely consistent with the
Raf activity assay in Fig. 2A and demonstrate that Ser364 in B-Raf is critical for inhibition by SGK.

*SGK stimulates phosphorylation of Ser364 in B-Raf*- Ser364 is highly conserved in Raf family kinases, including the C-elegans lin-45, and corresponds to Ser259 in C-Raf. Ser259 has been demonstrated to be a negative phosphorylation site. Phosphospecific antibody recognizing Ser259 of C-Raf is available. We found that this phosphospecific antibody can also recognize B-Raf. To further confirm that Ser364 in B-Raf is the target phosphorylation site of SGK, immunoblot of B-Raf was performed with the Ser259 phosphospecific antibody. Co-transfection of HA-SGK-S422D significantly increased the phosphorylation of Ser364 in B-Raf while the kinase inactive SGK had no effect at all (Fig. 2C, lanes 2-4). The recognition of the phosphospecific antibody is specific because substitution of Ser364 by an alanine residue completely eliminated the recognition by this antibody (Fig. 2C, lanes 6-8). Our observations confirm that Ser364 in B-Raf is a phosphorylation site by SGK.

*Differential selectivity of Akt and SGK towards FKHR*- The apparent high potency of SGK in inhibition of B-Raf activation could be due to the following two reasons. SGK may have a higher kinase activity than Akt. Alternatively, SGK may have higher substrate selectivity than Akt towards B-Raf. To compare the kinase activity of SGK and Akt, we performed *in vitro* kinase assays of SGK and Akt using a peptide substrate that has the consensus recognition sequence of both SGK and Akt. *In vitro* kinase assay demonstrated that wildtype SGK has a low basal activity while SGK-S422D displayed a much higher kinase activity (Fig. 3A). Akt-myr showed kinase activity similar to that of SGK-S422D when the peptide substrate was used (Fig. 3A). The *in vitro* kinase assays show that SGK-S422D has activity comparable with AKT-myr towards the peptide substrate, and suggest that SGK has a high substrate selectivity towards B-Raf.
The forkhead family transcription factors have been shown to be physiological targets of Akt. We have previously identified that FKHR can be phosphorylated and inhibited by Akt (44). To further compare the substrate selectivity of Akt and SGK, we examined the phosphorylation of recombinant FKHR fragments by immunoprecipitated Akt and SGK. FKHR fragments were expressed as GST fusion in E. coli and purified. Wildtype SGK displayed little activity towards the N-Terminal fragment of FKHR (Fig. 3B, lanes 5 and 6), which contains one Akt phosphorylation site Thr24. Similarly, SGK-S422D showed limited phosphorylation on the N-terminal fragment of FKHR (Fig. 3B, lanes 9, 10). In contrast, Akt-myr phosphorylated the N-terminal fragment of FKHR, albeit weak (Fig. 3B, lanes 17-20). The phosphorylation likely occurred on Thr24 because substitution of this residue by alanine eliminated the phosphorylation by either Akt or SGK (Fig. 3B, lane 10 and 18).

When the C-terminal fragment of FKHR, which contains two Akt consensus sites Ser256 and Ser319, was used in the kinase assay, some background phosphorylation was observed in the negative control experiments (lanes 3 and 4). However, SGK-S422D caused a considerable increase in phosphorylation of the C-terminal fragment of FKHR (Fig. 3B, lanes 11 and 12). The negative control of kinase dead SGKK127M mutant displayed no activity above the background (lanes 15 and 16). Interestingly, Akt was much more active towards the C-terminal fragment of FKHR than SGK-S422D (lanes 11 and19). Under similar conditions, phosphorylation of the C-terminal fragment by AKT was twice stronger than that by SGK. The phosphorylation of FKHR C-terminal fragment was eliminated if Ser256 and Ser319 were replaced by alanine residues (lanes 12 and 20). These observations demonstrate that the Akt consensus sites were utilized by both AKT and SGK. Furthermore, Akt is a more potent kinase towards FKHR than SGK.

Phosphorylation of FKHR results in a decrease of the transcriptional activity of FKHR. We compared the effect of Akt and SGK on FKHR activity in vivo. Expression of Akt-myr effectively inhibited the FKHR dependent transcription (Fig. 3C, lanes 1 and 4).
Similarly, expression of HA-SGK-S422D inhibited FKHR activity though less effective than Akt while the kinase inactive mutant SGK does not inhibit FKHR activity (Fig. 3C, lanes 2 and 3). We have shown that elimination of the Akt phosphorylation sites in FKHR dramatically increased the transcription activity of FKHR (44). The mutant FKHR-AAA molecule is not inhibited by Akt (Fig. 3C, lanes 5 and 7). Similarly, the transcription activity of FKHR-AAA is not inhibited by SGK (Fig. 3C, lane 6), supporting that SGK inhibits FKHR via phosphorylation of the Akt consensus sites. The above data in combination with results from figure 2 clearly demonstrate that Akt preferentially phosphorylates and inhibits FKHR while SGK is more active than Akt towards B-Raf.

Differential association of Akt and SGKT with FKHR and B-Raf: Direct protein-protein interaction has been found for many protein kinases and their respective substrates. We examined whether SGK and AKT showed a differential interaction with B-Raf and FKHR. GST-B-Raf was co-transfected with HA-SGK or HA-Akt in HEK293 cells. GST-B-Raf was purified by glutathione agarose resin and subjected to Western blotting with anti-HA for co-purified SGK or Akt. Figure 4A clearly shows that complex between SGK and B-Raf is more stable under these conditions than the interaction between Akt and B-Raf (Fig. 4A, the top panel) although both SGK and Akt were expressed at a similar level (the bottom panel). The association of SGK and Akt with B-Raf are specific because the negative control of GST co-precipitated neither Akt or SGK.

Similar co-immunoprecipitation was performed with FKHR. Flag-FKHR was co-expressed with HA-Akt or HA-SGK in HEK293 cells. Cell lysates were immunoprecipitated with anti-Flag to precipitate Flag-FKHR and Western blotted with anti-HA antibody for HA-SGK or HA-Akt. Results in figure 4B show that Akt preferentially associates with FKHR. To further test the association between SGK and B-Raf, immunoprecipitation of endogenous proteins were performed. SGK specific antibody precipitated B-Raf form un-transfected HEK293 cells (Fig. 4C, lane 2) while the control
serum did not precipitate SGK or B-Raf. These results confirm that SGK can form a complex with B-Raf in vivo. The preferential association between SGK and B-Raf are consistent with a strong inhibition of B-Raf by SGK.
Discussion

Recently, Akt has been implicated in negative regulation of B-Raf and C-Raf (33,34). Akt is reported to phosphorylate the CR2 region and inhibit Raf activity. SGK belongs to a new family of protein kinases which is closely related to Akt (39). SGK recognizes the RXRXXS/T consensus sequence (40,41). The biological function of SGK is not clear partly because few physiological substrates of SGK have been identified. Our results demonstrate that B-Raf is a potential substrate of SGK. Active SGK mutant effectively inhibits B-Raf activation in response to serum or Ras by phosphorylation of Ser364. Mutation of Ser364 by alanine renders B-Raf resistant to inhibition by SGK, indicating that phosphorylation of Ser364 is responsible for the inhibition of B-Raf activity. In contrast, mutation of other Akt consensus sites, Ser428 and Thr439, has no effect on the ability of B-Raf to be inhibited by SGK. In contrast to Akt, SGK can phosphorylate serine phosphoacceptor sites that do not have a bulky hydrophobic amino acid residue immediately C terminal to the phosphoacceptor residue (40). The sequence surrounding Ser364 match the preferred SGK phosphorylation sites, including an alanine at position 365 in B-Raf.

The relative specificity of SGK towards B-Raf is visible when compared to Akt and FKHR. SGK is more potent in B-Raf inhibition while Akt is more effective in FKHR inhibition. Protein-protein interaction data are consistent with the inhibition results and further support that B-Raf is a preferred substrate of SGK. The differential association between SGK and Raf likely contributes to the substrate selectivity of SGK towards B-Raf. Taken together, our data strongly indicate that SGK plays an important role in Raf regulation. Future studies of quantitative analysis of phosphorylation and detailed kinetic studies are required for further understanding the relative substrate specificity of AKT and SGK.

Akt and SGK are similarly activated by PDK1 and modulated by the PI3K pathway (40,41). Despite the similarity in the kinase domains of SGK and Akt, there are key
differences between the two kinases. Most notably, SGK does not have the PH domain, which is present in the N-terminal region of Akt. The two kinases recognize similar but not identical consensus sites in peptide substrates. Akt phosphorylates B-Raf presumably on both Ser364 and Ser428 while SGK likely phosphorylates on Ser346, indicating that the two kinases have overlapping but not identical recognition sites in B-Raf. Similar observation has been made in FKHRL1, a transcription factor closely related to FKHR. SGK displays a preference for Ser315 in FKHRL1 whereas Akt favors Ser253 (43). In addition, SGK is regulated at both transcriptional and post-translational levels (39,45). The expression of SGK is rapidly induced by a variety of stimuli, including serum, steroid hormones, cytokines, and osmotic stress (39,46-48). These differences suggest that Akt and SGK may have complementary rather than redundant functions. We propose that both SGK and Akt play a role in negative regulation of Raf, with SGK being a more potent inhibitor. The fact that SGK is induced by serum and growth factors indicates that SGK may constitute a negative feedback loop to suppress constitutive activation of the Raf-MEK-ERK kinase cascade.

Protein kinase A is known to phosphorylate and inhibit Raf activity. In C-Raf, PKA is reported to phosphorylate Ser43 and inhibit C-Raf activation (29,32). However, recent results dispute the role of Ser43 in regulation of C-Raf by PKA, and indicate that Ser43 is not required for C-Raf inhibition by PKA (49). Furthermore, Ser43 is not conserved in B-Raf yet B-Raf is also negatively regulated by PKA. The effect of cAMP on B-Raf regulation is rather complex because cAMP can also induce B-Raf activation in some cells such as PC12 (50). Thus, the mechanism of Raf inhibition by cAMP is not completely understood. Interestingly, SGK is activated by intracellular cAMP (48). Therefore, SGK may mediate the inhibitory effects of cAMP on Raf activity. Regulation of SGK by cAMP could provide an important mechanism for cross-talk between signal transductions of the trimeric G-protein coupled serpentine receptors and the Ras coupled tyrosine kinase receptors.
The SGK phosphorylation site Ser364 in B-Raf is conserved in C-Raf, Drosophila Raf, and the C. elegans lin-45 Raf. These conservations suggest that SGK will likely play a role in negative regulation of all members of the Raf family kinases. We have observed that the corresponding sites in lin-45 Raf indeed play a negative role in lin-45 function \textit{in vivo}. Substitution of the Akt/SGK recognition site by alanine results in an active lin-45 Raf and induces multi-vulvae phenotypes (Chong et al, unpublished observations). Interestingly, C. elegans contains a single SGK predicted by the genome sequence. The fact that SGK is regulated by a wide variety of extracellular stimuli provides a point of signal integration. Regulation of SGK at either transcriptional or posttranslational level represents a possible mechanism of cross-talk between other signaling pathways and the Raf-ERK pathway.
References

1. English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M. H. (1999) *Exp Cell Res* **253**(1), 255-70
2. Hagemann, C., and Rapp, U. R. (1999) *Exp Cell Res* **253**(1), 34-46
3. Marshall, C. J. (1995) *Cell* **80**(2), 179-85
4. Morrison, D. K., and Cutler, R. E. (1997) *Curr Opin Cell Biol* **9**(2), 174-9
5. Kolch, W. (2000) *Biochem J* **351** Pt 2, 289-305.
6. Vojtek, A. B., and Der, C. J. (1998) *J Biol Chem* **273**(32), 19925-8
7. Robinson, M. J., and Cobb, M. H. (1997) *Curr Opin Cell Biol* **9**(2), 180-6.
8. Chang, L., and Karin, M. (2001) *Nature* **410**(6824), 37-40.
9. Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowtiz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991) *Embo J* **10**(4), 885-92
10. Alessi, D. R., and Cohen, P. (1998) *Curr Opin Genet Dev* **8**(1), 55-62.
11. Resing, K. A., Mansour, S. J., Hermann, A. S., Johnson, R. S., Candia, J. M., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1995) *Biochemistry* **34**(8), 2610-20.
12. Zheng, C. F., and Guan, K. L. (1994) *Embo J* **13**(5), 1123-31
13. Magnuson, N. S., Beck, T., Vahidi, H., Hahn, H., Smola, U., and Rapp, U. R. (1994) *Semin Cancer Biol* **5**(4), 247-53
14. Daum, G., Eisenmann-Tappe, I., Fries, H. W., Troppmair, J., and Rapp, U. R. (1994) *Trends Biochem Sci* **19**(11), 474-80
15. Gorman, C., Skinner, R. H., Skelly, J. V., Neidle, S., and Lowe, P. N. (1996) *J Biol Chem* **271**(12), 6713-9
16. Mott, H. R., Carpenter, J. W., Zhong, S., Ghosh, S., Bell, R. M., and Campbell, S. L. (1996) *Proc Natl Acad Sci U S A* **93**(16), 8312-7
17. Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995) *Nature* **375**(6532), 554-60
18. Vojtek, A. B., and Cooper, J. A. (1993) *J Cell Sci* **105**(Pt 3), 777-85

19. Cutler, R. E., Jr., Stephens, R. M., Saracino, M. R., and Morrison, D. K. (1998) *Proc Natl Acad Sci USA* **95**(16), 9214-9

20. Jansen, H. W., Lurz, R., Bister, K., Bonner, T. I., Mark, G. E., and Rapp, U. R. (1984) *Nature* **307**(5948), 281-4.

21. Dent, P., Jelinek, T., Morrison, D. K., Weber, M. J., and Sturgill, T. W. (1995) *Science* **268**(5219), 1902-6.

22. Stokoe, D., and McCormick, F. (1997) *Embo J* **16**(9), 2384-96

23. Tamada, M., Hu, C. D., Kariya, K., Okada, T., and Kataoka, T. (1997) *Oncogene* **15**(24), 2959-64.

24. Zhang, X. F., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) *Nature* **364**(6435), 308-13.

25. Barnard, D., Diaz, B., Clawson, D., and Marshall, M. (1998) *Oncogene* **17**(12), 1539-47.

26. King, A. J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S., and Marshall, M. S. (1998) *Nature* **396**(6707), 180-3.

27. Mason, C. S., Springer, C. J., Cooper, R. G., Superti-Furga, G., Marshall, C. J., and Marais, R. (1999) *Embo J* **18**(8), 2137-48

28. Zhang, B. H., and Guan, K. L. (2000) *Embo J* **19**(20), 5429-39.

29. Cook, S. J., and McCormick, F. (1993) *Science* **262**(5136), 1069-72

30. Morrison, D. K., Heidecker, G., Rapp, U. R., and Copeland, T. D. (1993) *J Biol Chem* **268**(23), 17309-16.

31. Schramm, K., Niehof, M., Radziwill, G., Rommel, C., and Moelling, K. (1994) *Biochem Biophys Res Commun* **201**(2), 740-7

32. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1993) *Science* **262**(5136), 1065-9
33. Zimmermann, S., and Moelling, K. (1999) *Science* **286**(5445), 1741-4.

34. Guan, K. L., Figueroa, C., Brtva, T. R., Zhu, T., Taylor, J., Barber, T. D., and Vojtek, A. B. (2000) *J Biol Chem* **275**(35), 27354-9.

35. Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997) *Cell* **88**(4), 435-7.

36. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) *Genes Dev* **13**(22), 2905-27.

37. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) *Curr Biol* **7**(4), 261-9.

38. Toker, A., and Newton, A. C. (2000) *J Biol Chem* **275**(12), 8271-4.

39. Webster, M. K., Goya, L., Ge, Y., Maiyar, A. C., and Firestone, G. L. (1993) *Mol Cell Biol* **13**(4), 2031-40.

40. Kobayashi, T., Deak, M., Morrice, N., and Cohen, P. (1999) *Biochem J* **344** Pt 1, 189-97.

41. Park, J., Leong, M. L., Buse, P., Maiyar, A. C., Firestone, G. L., and Hemmings, B. A. (1999) *Embo J* **18**(11), 3024-33.

42. Prasad, N., Topping, R. S., Zhou, D., and Decker, S. J. (2000) *Biochemistry* **39**(23), 6929-35.

43. Brunet, A., Park, J., Tran, H., Hu, L. S., Hemmings, B. A., and Greenberg, M. E. (2001) *Mol Cell Biol* **21**(3), 952-65.

44. Tang, E. D., Nunez, G., Barr, F. G., and Guan, K. L. (1999) *J Biol Chem* **274**(24), 16741-6.

45. Buse, P., Tran, S. H., Luther, E., Phu, P. T., Aponte, G. W., and Firestone, G. L. (1999) *J Biol Chem* **274**(11), 7253-63.

46. Bell, L. M., Leong, M. L., Kim, B., Wang, E., Park, J., Hemmings, B. A., and Firestone, G. L. (2000) *J Biol Chem* **275**(33), 25262-72.

47. Gonzalez-Robayna, I. J., Falender, A. E., Ochsner, S., Firestone, G. L., and Richards, J. S. (2000) *Mol Endocrinol* **14**(8), 1283-300.
48. Perrotti, N., He, R. A., Phillips, S. A., Haft, C. R., and Taylor, S. I. (2001) *J Biol Chem* **276**(12), 9406-9412.

49. Sidovar, M. F., Kozlowski, P., Lee, J. W., Collins, M. A., He, Y., and Graves, L. M. (2000) *J Biol Chem* **275**(37), 28688-94.

50. Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Rim, C. S., and Stork, P. J. (1997) *Cell* **89**(1), 73-82
**Abbreviation**- SGK, serum- and glucocorticoid-induced protein kinase; PKA, protein kinase A; MAPK, mitogen activated protein kinase; ERK, extracellular signal regulated kinase; MEK, MAPK and ERK kinase; PMA, phorbol 12-myristate 13-acetate; PH, pleckstrin homology domain; PKB, protein kinase B; PDK, phospholipid dependent kinase; PI3K, phosphoinositide 3-kinase; GSK3, glycogen synthase kinase-3, IP, immunoprecipitation; WB, Western blot.

**Acknowledgements**- We wish to thank Dr. Tom Lanigan for critical reading of the manuscript.; Drs. Stuart Decker and Roman Herrera for SGK plasmids. B.H.Z is a recipient of *C. J. Martin* Postdoctoral Fellowship from Australian National Health and Medical Research Council (NHMRC). This work was supported by National Institutes of Health (NIH) Grants, Walther Cancer Institute, and the MacArthur Fellowship to K.L.G.
Figure legends

Figure 1. SGK inhibits Ras- or serum-induced B-Raf activation and displays preference for B-Raf regulation as compared with Akt.

HEK293 cells were transfected with vector, wildtype B-Raf (HA-B-Raf-WT) or co-transfected with constitutively active HRasV12, wildtype SGK (SGK-WT), constitutively active SGK (HA-SGK-S422D), kinase dead SGK(HA-SGK-K127M) or constitutively active Akt (HA-Akt-myr), as indicated. After overnight serum starvation, cells (lanes 8-11) were treated with10% serum for 15 min. B-Raf was immunoprecipitated from lysates and Raf activity was assessed by an *in vitro*-coupled kinase assay in which recombinant MEK, ERK and the C-terminal fragment of Elk-1 were used. Raf activity (top panel) was detected by phosphorylation of Elk-1 (^32P-incorporation), which depends on the *in vitro* activation of ERK in the reactions. Quantitation of relative Raf activity is indicated underneath each lane of the top panel. Western blots of HA-B-Raf proteins used for the *in vitro* kinase assay and HA-SGK/Akt in cell lysates are shown in the middle panel and bottom panel, respectively.

Figure 2. Ser364 of B-Raf is a key target site for inhibition by SGK.

A, Inhibition of B-Raf activity by SGK requires Ser364 in B-Raf. Experiments were performed similar to those in figure 1. HA-B-Raf-A and HA-B-Raf-AA contain alanine substitutions of residue 364, 428 and 439, respectively. HA-B-Raf-AAA contains alanine substitution of Ser364, Ser428 and Thr439. Quantitation of Elk1 phosphorylation is indicated below the pElk1 panel.

B, SGK inhibits the activation of ERK by B-Raf in transfected HEK293 cells. Myc-ERK1 was co-transfected with various B-Raf mutants into HEK293 cells. Co-transfections with Akt or SGK are indicated. The myc-ERK1 was immunoprecipitated and kinase activity was assayed directly using GST-Elk-1 as a substrate. The ERK kinase activity (phosphorylation of Elk-1) is shown in the top panel while the amounts of ERK protein in
kinase assays are shown in the bottom panel. Expression of B-Raf in cell lysates is detected by anti-HA Western blot. Expression of Akt and SGK, both having similar molecular weight, are also presented. Quantitation of Elk1 phosphorylation is indicated below the pElk1 panel.

C, SGK stimulates Ser364 phosphorylation in B-Raf. B-Raf wild type (WT) or Ser364Ala mutant (B-RafA) was co-transfected with SGK or Akt as indicated. B-Raf was immunoprecipitated and Western blotted with anti-phospho-Ser364 specific antibody (top panel). The expression levels of B-Raf and SGK/Akt are shown in the middle and the bottom panels, respectively.

**Figure 3.** SGK exhibits lower activity to phosphorylate FKHR than Akt.

A, *In vitro* kinase activity of SGK and Akt using peptide substrate. SGK and Akt were transfected into HEK293 cells. The proteins were immunoprecipitated and kinase activity was assayed using a peptide substrate (see Materials and Methods). Kinase activities were measured by incorporation of $^{32}$P into substrates. Similar amounts of protein were used in kinase assays for SGK and Akt. Vector transfection was included as a background control.

B, *In vitro* phosphorylation of FKHR by SGK and Akt. Immunoprecipitated Akt or SGK were assayed *in vitro* using purified GST fusion of the N-terminal fragment or the C-terminal fragment of FKHR as substrate. Phosphorylation of FKHR fragments is shown in the upper panel. N denotes the N-terminus of wild type FKHR while N’ denotes the same N-terminal fragment containing T24A mutation. Similarly, C and C’ denote the wild type and S256A/S319A mutant of the C-terminal fragment of FKHR, respectively. Kinase proteins used in phosphorylation assays are shown in the lower panel as indicated. Quantitation of phosphorylation of the C-terminal fragment of FKHR is indicated underneath the upper panel for lanes 7, 11, 15, and 19. The value for wild type SGK was arbitrary set as one.
C. Inhibition of FKHR transcriptional activity by SGK requires the intact phosphoacceptor sites. A FKHR reporter was co-transfected with various forms of SGK and wild type (lanes 1-4) or the constitutively active FKHR mutant (lane 5-7). FKHR-AAA has serine to alanine substitutions at all three putative Akt phosphorylation sites and is constitutively active. The relative luciferase activity is measured and normalized with co-transfected β-galactosidase internal control. Error bars are derived from three independent duplicated experiments. Expression levels of SGK and Akt are shown in the bottom panel.

Figure 4. SGK and Akt display differential preference in interaction with B-Raf and FKHR.

A. SGK preferentially binds to B-Raf. Interactions between GST-B-Raf and SGK or Akt were examined. HEK293 cells were transfected with GST-B-Raf and HA-Akt or HA-SGK. GST vector was included as controls. GST-B-Raf was purified by glutathione agarose resin. The co-purified SGK and Akt were detected by anti-HA immunoblot (the top panel). Expression of Akt and SGK in cell lysates is shown in the bottom panel. The GST controls and GST-B-Raf in cell lysates and in purified form are shown in the two middle panels. IP and WB denote immunoprecipitation and Western blot, respectively.

B. Akt associates preferentially with FKHR. Flag-tagged FKHR was co-transfected with Akt or SGK as indicated. Samples were immunoprecipitated by anti-HA antibody and blotted with anti-Flag. The co-immunoprecipitated Akt and SGK are shown in the top panel.

C. Endogenous SGK associates with B-Raf. HEK293 cell lysates were immunoprecipitated with anti-SGK antibody followed by Western blotting with anti-B-Raf antibody. B-Raf is co-immunoprecipitated by anti-SGK antibody (lane 2) but not by the control serum (lane 1).
|                  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|------------------|---|---|---|---|---|---|---|---|---|----|----|
| 10% serum (15 min)| - | - | - | - | - | - | + | + | + | +  | +  |
| HRasV12          | - | - | - | + | + | + | + | - | -  | -  | -  |
| HA-SGK-WT        | - | - | - | - | - | - | - | - | +  | -  | -  |
| HA-SGK-S422D     | - | - | + | - | + | - | - | - | -  | -  | -  |
| HA-SGK-K127M     | - | - | - | - | + | - | - | - | +  | -  | -  |
| HA-Akt-myr       | - | - | - | - | - | + | - | - | -  | +  | -  |
| HA-B-Raf-WT      | - | + | + | + | + | + | + | + | +  | +  | +  |

**Fig. 1**

- **pElk**
  - Activity: 1 3.6 1.2 14 5 12 7.1 8.5 2.1 8.7 6.1

- **HA-B-Raf**
  - Activity: 1 3.6 1.2 14 5 12 7.1 8.5 2.1 8.7 6.1

- **HA-SGK/Akt**
  - Activity: 1 3.6 1.2 14 5 12 7.1 8.5 2.1 8.7 6.1
Fig. 3

A

| Treatment          | SGK/Akt activity (cpm) |
|--------------------|-------------------------|
| Vector             | 10,000                  |
| HA-SGK-WT          | 20,000                  |
| HA-SGK-S422D       | 30,000                  |
| HA-SGK-K127M       | 40,000                  |
| HA-Akt-myr         | 10,000                  |

B

| Treatment          | pFKHR Activity          |
|--------------------|--------------------------|
| HA-SGK/Akt         | + + + + - - - - - - - - |
| FKHR-WT            | + + + + + + + + + + + +  |
| FKHR-AAA           | - - - - + + + + + + + +  |
| HA-SGK-S422D       | - - - - + + + + + + + +  |
| HA-SGK-K127M       | - - - - + + + + + + + +  |
| HA-Akt-myr         | - - - - + + + + + + + +  |

C

| Relative luciferase activity |
|------------------------------|
| 1   2   3   4   5   6   7 |
| HA-SGK/Akt                   | + + + + - - - - - - - - |
| FKHR-WT                      | + + + + + + + + + + + +  |
| FKHR-AAA                     | - - - - + + + + + + + +  |
| HA-SGK-S422D                 | - - - - + + + + + + + +  |
| HA-SGK-K127M                 | - - - - + + + + + + + +  |
| HA-Akt-myr                   | - - - - + + + + + + + +  |
Fig. 4

A

IP: GST
WB: HA

IP: GST
WB: GST

Lysate
WB: GST

Lysate
WB: HA

Vector
GST-B-Raf
HA-SGK
HA-Akt

1 2 3 4

+ - + -

- + - +

+ + - -

B

IP: HA
WB: Flag

IP: HA
WB: HA

Lysate
WB: Flag

Lysate
WB: HA

Vector
Flag-FKHR
HA-SGK/Akt
HA-SGK/Ark
HA-Akt

1 2 3 4

+ - + -

+ + + +

- + - -

C

IP: SGK
WB: B-Raf

Lysate
WB: SGK

Lysate
WB: B-Raf

Control
1 2 IP: SGK

- + - +

+ + - +

- - + +
Serum and glucocorticoid-inducible kinase SGK phosphorylates and negatively regulates B-Raf

Bao-Hong Zhang, Eric Tang, Tianqing Zhu, Michael Greenberg, Anne Vojtek and Kun-Liang Guan

J. Biol. Chem. published online June 15, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102808200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts