J. Biochem. **133**, 103–108 (2003)
DOI: 10.1093/jb/mvg010

Inhibition of Mitogen-Activated Kinase Kinase Kinase 3 Activity through Phosphorylation by the Serum- and Glucocorticoid-Induced Kinase 1

Jaesun Chun[1], Taegun Kwon[3], Dae Joong Kim[3], Ingun Park[1], Guhung Chung[5], Eun Jeoung Lee[1], Soon Kwang Hong[4], Soo-Ik Chang[2], Hack Young Kim[2] and Sang Sun Kang[*,1]

[1]School of Science Education and [2]School of Biological Science, Chungbuk National University, Chongju, 361-763, Republic of Korea; [3]Samsung Biomedical Research Institute, Seoul, 161-763, Republic of Korea; [4]Division of Life Science, Myungji University, Youngin, Kyungki do, 361-763, Republic of Korea; and [5]School of Biological Science, Seoul National University, Seoul, 151-742, Republic of Korea

Received October 2, 2002; accepted October 28, 2002

The mitogen-activated protein kinase kinase kinase 3 (MEKK3) is a member of the MAP kinase family whose cellular activity is elevated in response to growth factors, oxidative stress, and hyperosmolar conditions. MEKK3 regulates MKK3 and MKK5/6/7. MEKK3 is involved distinctively in the signal pathway for blocking cell proliferation and cell cycle progression, contradictory to the biological responses commonly associated with other members of MEKKs. Based information concerning the substrate specificity of serum- and glucocorticoid-induced kinase 1 (SGK1), R-x-R-x-x-(S/T)-ϕ, where ϕ indicates a hydrophobic amino acid, two putative phosphorylation sites (Ser^{166} and Ser^{337}) were found in MEKK3. It was shown that the recombinant MEKK3 protein and fluorescein-labeled MEKK3 peptides (FITC-^{159}epRsRhlSVi^{168} and FITC-^{330}dpRgRlpSAd^{339}) are phosphorylated by SGK1 *in vitro*. It was also observed that the intrinsic kinase activity of MEKK3 on Ser^{189} of MKK3 (equivalent to Ser^{207} of MKK6) decreased along with phosphorylation of Ser^{166} and Ser^{337} in MEKK3 *in vitro* and *in vivo*. Therefore, it is suggested that SGK1 inhibits MEKK3-MKK3/6 signal transduction by phosphorylation of MEKK3.

Key words: 14–3-3, MEKK3, MKK6, SGK1.

The mitogen-activated protein kinase kinase kinase 3 (MEKK3) has been identified as one of the upstream regulatory components of MAPK cascades in several systems (*1–4*). The kinase domain of MEKK3 is found in the carboxy-terminal part of the enzume. MEKK2 and MEKK3 are markedly homologous (94%) in their kinase domains (*1*). Both MEKK2 and MEKK3 are able to activate SAPK pathway *in vivo*, and their amino-terminal moieties have no significant motif that would suggest a defined regulatory function, such as the PH, SH3, CRIB, RB or proline-rich domains of MEKK1/2 (*2*, *3*). Thus, the upstream signal cascade of MEKK2/3 remains to be characterized. It has been reported that MEKK2/3 is distinct from the other MEKK family members, MEKK 1/4/5 (*3*, *4*). MEKK3 activates MKK3/6 through phosphorylation of specific Ser/Thr residues in their VIII domains (*1–4*). In turn, MKK3 specifically phosphorylates and activates SAPK, and MKK6 phosphorylates and activates both SAPK1 and SAPK2 (*5–7*). MEKK3 is also likely to prevent SAPK2 activation but to allow SAPK1 activation (*1–3*, *8*). Thus, MEKK3 seems to be involved in the signal pathway for blocking cell proliferation and cell cycle progression, in contrast to the biological responses commonly associated with other MEKKs (*2*, *8*).

The serum- and glucocorticoid-induced kinase 1 (SGK1) was identified as a Ser/Thr protein kinase. SGK1 is regulated transcriptionally by serum, glucocorticoids and corticosteroids in several cell types (*9–11*). Recent findings suggest that SGK1 is an important gene in the early action of corticosteroids on epithelial sodium reabsorption (*12*). SGK1 shares 45–55% homology with the catalytic domain of protein kinase C, cAMP-dependent protein kinase A, Akt1, and the p70 ribosomal protein S6 kinase (*9*, *13*). It has been well documented that the expression of SGK1 is high in the thymus, lung, and ovary and low but detectable in mammary gland and several other tissues in adult rats (*14*, *15*). It has been reported that human SGK1 is not transcriptionally regulated by corticosteroids in a hepatoma cell line, and thus far no glucocorticoid response element has been identified in the human SGK1 gene (*16*).

SGK2 and SGK3 were characterized by gene homology search (*17*). In addition, cytokine-independent survival kinase (CISK) has been identified as a mouse homologue of human SGK3 by the new large-scale mammalian genetic screening method (*18*). It has been reported that the activation of all SGKs requires the phosphorylation of both Thr^{263} and Ser^{422} by 3-phosphoinositide–dependent protein kinase–1 (PDK1), similar to the Akt1 activation mechanism (*17*, *19*). However, SGK1 does not contain a pleckstrin homology (PH) domain where the PI 3 kinase lipid products bind in order to regulate the translocation

[*]To whom correspondence should be addressed. Tel: +82-43-261-3278, Fax: +82-43-271-0526, E- mail: jin95324@cbucc.chungbuk.ac.kr

of Akt1 into the plasma membrane for PDK1 phosphorylation (*17*, *19*, *20*). Thus, it is presently unknown how the localization of SGK1 to the plasma membrane is regulated. Xu *et al*. demonstrated that CISK/SGK3 contains a Phox homology (PX) domain that interacts with phosphatidylinositol (*18*, *21*). They suggested that the PX domain regulates the translocation and function of CISK through its direct interaction with phosphoinositides. Thus, it seems to be that CISK and SGK3 utilize different lipid binding domains to accomplish a similar mechanism of activation in response to PI-3 kinase signaling.

SGK1 has also been identified as promoting cell survival, the same function as for Akt1 (*17*, *19*, *22*). However, the authentic substrate protein of SGK1 has been characterized in only a few cases up to now. Brunet *et al.* reported that SGK1 phosphorylates Forkhead transcription factors, such as FKHRL1, causing FKHRL1 to exit from the nucleus, so that the target genes of FKHRL1 are turned off, resulting in the promotion of cell survival in part by the phosphorylation and inactivation of FKHRL1 (*23*). The same authors indicated that SGK1 plays a role in concert with Akt1 in propogating the effects of PI3K activation, including cell survival and cell cycle progression. Zhang *et al.* reported that SGK1 phosphorylates and down-regulates B-Raf1 (*24*).

The substrate protein specificity of SGKs is also known to be similar to that of Akt1 (*17*, *19*, *23*, *24*). The arginine residues at positions –5 and –3 are conserved relative to the serine/threonine residues that are phosphorylated in these proteins (R-x-R-x-x-(S/T)-ϕ; ϕ indicates a hydrophobic amino acid). Thus, proteins containing this amino acid sequence seems to be potent substrate proteins for SGK1 and Akt1. However, it remains to be demonstrated whether the substrate proteins for Akt1 and SGK1 are the same or not.

From the SGK1 substrate consensus sequence information, we noticed putative SGK1 phosporylation sites in MEKK3 (159epRsRhlSVi168 and 330dpRgRlpSAd339), but not in other MEKKs (*1–3*). Recently, Gratton *et al*. demonstrated in a co-transfection experiment that Akt1 down-regulates MEKK3 through phosphorylation (*25*). Therefore, we investigated whether the phosphorylation of Ser^{166} and Ser^{337} in MEKK3 affects the intrinsic kinase activity of MEKK3 *in vivo* and *in vitro*. It was observed that two synthetic MEKK3 peptides (159epRsRhlSVi168 and 330dpRgRlpSAd339) are phosphorylated by SGK1, and that the kinase activity of the phosphorylated MEKK3 is reduced. These observations suggest that MEKK3 is regulated by SGK1 as one of the physiological substrates of SGK1.

MATERIALS AND METHODS

Cell Culture—COS-1 was purchased from ATCC (Manassas, VA). Media and supplements were obtained from GIBCO (Grand Island, NY). The cell line was maintained in Dulbecco's Modified Essential Medium (DMEM) containing 10% heat inactivated (for 30 min at 56°C) fetal bovine serum (FBS), 100 U potassium penicillin/ml, 100 μg streptomycin/ml, 2 mM glutamine and 20 mM sodium bicarbonate. The cells were incubated in an atmosphere of 5% CO_2, 95% humidity at 37°C. The growth medium was changed every 3 days.

Antibodies—Monoclonal antibodies against the hexahistidine (His_6) epitope and GFP were purchased from Calbiochem (Emeryville, CA). Antibodies against MKK3 and MEKK3 were purchased from StressGene (Victoria, British Columbia, Canada). SGK1 protein was purchased from Upstate Biochem (New York, NY), and the antibody against SGK1 was purchased from New England Biolabs (Beverly, MA).

Assays of SGK1 and MEKK3—The kinase activities of SGK1 and MEKK3 were assayed by the protocol provided by Promega (Wisconsin, USA) with the PepTag Non-radioactive Protein Kinase C (PKC) Assay System, except for the substrate peptides (*26*, *27*). MEKK3 peptides (FITC-^{159}epRsRhlSVi^{168} and FITC-^{330}dpRgRlpSAd^{339}) were used as substrates for SGK1 kinase assay, and fluorescein-conjugated MKK3 peptide (FITC-^{183}SGYLVDSVAKTIA^{195}) was used as a substrate for MEKK3 kinase assay, and were purchased from Peptron (Daejun, Korea). 0.5 μg of fluorescein-labeled oligopeptide was incubated with 10 μl of differentially-treated cell lysates, with the activated MEKK3 or SGK1 in 25 μl of protein kinase reaction buffer (20 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol, 0.2 mM EGTA, 20 μM ATP, 1 μg phosphatidylserine) at 30°C for 30 min. The reactions were stopped by heating at 95°C for 10 min. The phosphorylated peptides were separated in 0.8% agarose gels at 100 V for 15 min. The phosphorylated products which gained one more negative charge, migrated to the anode. After electrophoresis, the gel was photographed on a transilluminator. The optical density of the phosphorylated product was measured by densitometry.

Site-Directed Mutagenesis of MEKK3 and MKK3—Both Ser^{166} and Ser^{337} in MEKK3 (a gift from Dr. Schenbelist) were replaced with alanine to generate a mutant MEKK3 S166A S337A construct, using a "Chameleon" double-stranded, site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Ser^{189} of MKK3 (a gift from Dr. Han) was also mutated to alanine with the same mutagenesis kit to make mutant MKK3 S189A. The mutations were confirmed by DNA sequencing.

MEKK3 Expression Vector Transfection and Purification—Mutagenized and wild type human MEKK3 were cloned with GFP-tagged mammalian expression vector (InVitrogen) after PCR with the forward primer; 5′-atggacgaacaggaggcattg-3′, and the reverse primer; 5′-gagagctcagtacatgagctg-3′. For mammalian expression, GFP-MEKK3 constructs, or GST-SGK1 (a gift from Dr. Alessi) was transfected or co-transfected into COS-1 cells by the lipofectin transfection method (Gibco-BRL). The transfected cells (2×10^7) were lysed in RIPA lysis buffer, and 500 μl aliquots of the cleaned lysates were incubated with anti-GFP antibody, and precipitated with protein A agarose beads. The beads were washed three times with excess cell lysis buffer. The final pellet was used for SGK1 or MEKK3 kinase assays as described above.

Expression and Purification of Recombinant Proteins—The wild type (wt) MEKK3 and the double mutant (dm) MEKK3 S166A S337A were cloned with prokaryotic expression vector, pTrcHis2-TOPO (InVitrogen), after PCR with the same primer set used to generate mammalian expression constructs. The wild type MKK3 (wt) and MKK S169A were cloned into pTrcHis2-TOPO vector after PCR with the forward primer; 5′-atgtccaagccacccg-

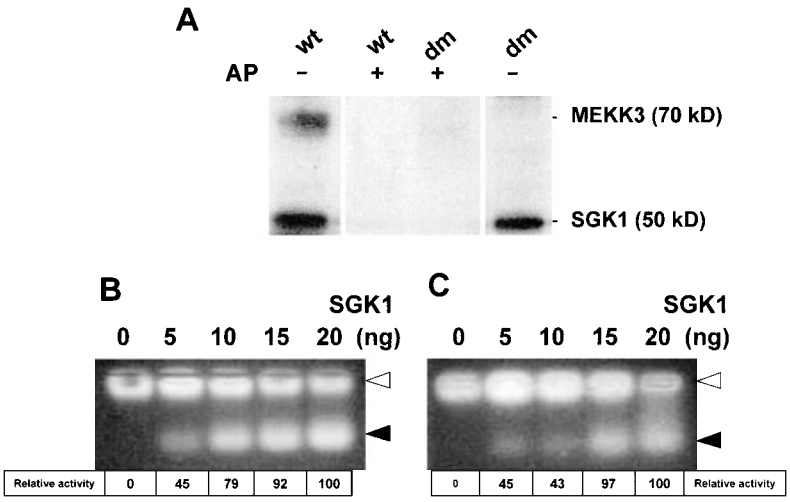

Fig. 1. **SGK1 phosphorylates recombinant MEKK3 proteins and MEKK3 peptides.** To determine whether SGK1 phosphorylates MEKK3, SGK1 was incubated in the presence of [γ-^{32}P]ATP with wild type (wt) MEKK3 and the double mutant (dm) MEKK3 S166AS337A purified from recombinant *E. coli*. Alkaline phosphatase (AP) was added to confirm the phosphorylation of the proteins. The phosphorylated MEKK3 and SGK1 are indicated in (A). To further demonstrate that SGK1 phosphorylates MEKK3, the phosphorylation of two fluorescein-conjugated MEKK3 peptides (FITC-^{159}epRsRhlSVi^{168} and FITC-^{330}dpRgRlpSAd^{339}) was measured as described in Materials and Methods. The phosphorylation of two MEKK3 peptides, FITC-^{159}epRsRhlSVi^{168} (B) and FITC-^{330}dp*RgR*lpSAd^{339} (C), increased depending on the concentration of SGK1. The phosphorylated product (solid arrowheads) and the unphosphorylated substrate (open arrowheads) are indicated. All the figures in this article show the results of four experiments repeated independently. The mean relative activity is indicated below.

cacc-3′ and the reverse primer; 5′-cagcccctatgatgtcttctc-3′. The hexahistidine-tagged proteins were expressed in *Escherichia coli* BL21 and purified with Ni^{2+}-NTA agarose beads according to the manufacturer's instructions. The purified proteins were used for SGK1 or MEKK3 protein kinase assay.

Phosphorylation of Recombinant MEKK3 or MKK3—The preheated inactive MEKK3 (wt), MEKK3 S166A S337A (dm) protein tagged with hexahistidine (30 pmol), was incubated with active SGK1 (0.5 μg, from Upstate Biotechnology, New York, USA) in 50 μl protein kinase assay buffer (20 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 0.2 mM EGTA, 20 μM ATP, 1 μCi [γ-^{32}P]ATP when indicated, and 5 μg phosphatidylserine) for 1 h at 30°C. To control protein phosphorylation, the phosphorylated reaction mixture was treated with 10 ng of alkaline phosphatase for 30 min at 30°C. Preheated inactive His_6-MKK3 (wt) or His_6-MKK S169A (30 pmol) was incubated with active bacterially-expressed MEKK3 in 50 μl of protein kinase assay buffer, as for the above SGK1 assay. The reaction mixture was loaded onto a 10% SDS-PAGE gel, and the dried gel was exposed to X-ray film.

Immunoprecipitation and In Vitro SGK1 or MEKK3 Kinase Assays—Cells were solubilized in lysis buffer (20 mM HEPES, pH 7.6, 1% Triton X-100, 137 mM NaCl, 0.1 mM Na_3VO_4, 25 mM β-glycerophosphate, 3 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) for 10 min at 4°C. The cell lysates were then centrifuged at 15,000 ×*g* for 15 min at 4°C and the supernatants were incubated with anti-GST or anti-GFP antibody for 16 h at 4°C. The immuno-complex was precipitated with protein A agarose beads (Upstate Biochem, New York, NY). The beads were washed twice with 1 ml of lysis buffer and then twice more with 0.5 ml of kinase reaction buffer (20 mM HEPES pH 7.6, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, 25 mM β-glycerophosphate, and 2 mM dithiothreitol). The protein kinase assays were performed and analyzed as described above.

RESULTS AND DISCUSSION

The Phosphorylation of MEKK3 by SGK1—It has been shown that SGK1 recognizes certain amino acid sequences (R-x-R-x-x-(S/T)-ϕ; ϕ indicates a hydrophobic amino acid) in its substrate proteins, similar to Akt1 (*17*, *19*, *23*, *24*). The existence of two putative SGK1 phosporylation sites in MEKK3 (159epRsRhlSVi168 and 330dpRgRlpSAd339) led us to suspect that SGK1 phosphorylates MEKK3, an important regulatory protein in SAPK signal transduction.

To demonstrate whether SGK1 phosphorylates MEKK3, SGK1 was incubated with recombinant MEKK3 proteins or peptides *in vitro*. As shown in Fig. 1A, the recombinant wild type MEKK3 protein was phosphorylated by SGK1. The mutant MEKK3 S166A S337A, in which both Ser^{166} and Ser^{337} are replaced by alanine, was not phosphorylated by SGK1. To confirm the phosphorylation, the reaction mixture was treated with alkaline phosphatase and the phosphorylated protein bands disappeared (Fig. 1A, middle lanes).

Further, to determine whether Ser^{166} and Ser^{337} in MEKK3 are phosphorylated by SGK1, the fluorescein-labeled MEKK3 peptides (FITC-^{159}epRsRhlSVi^{168} and FITC-^{330}dpRgRlpSAd^{339}) were incubated with SGK1. As shown in Fig. 1B and C, the amount of phosphorylated MEKK3 peptide increased depending on the SGK1 concentration. There was no detectable amount of phosphorylated product at 0 min (Fig. 1, B and C). Thus these observations demonstrate that SGK1 phosphorylates Ser^{166} and Ser^{337} in MEKK3.

According to the reported substrate preference of Akt1 and SGK1, and our *in vitro* data (Fig. 1 B and C), Ser^{166} is more easily phosphorylated than Ser^{337} by SGK1. However, it is unclear whether this is also true *in vivo* because SGK1 and Akt1 may display differences in the efficiency of phosphorylation on the two regulatory sites of MEKK3, similar to the phosphorylation of B-Raf or FKHRL1 (*23*, *24*).

The Phosphorylation of MKK3 Peptide by MEKK3—It is known that MEKK3 phosphorylates Ser^{189} on MKK3 and Ser^{207} on MKK6 (*1–3*, *8*). As shown in Fig. 2A, the wild

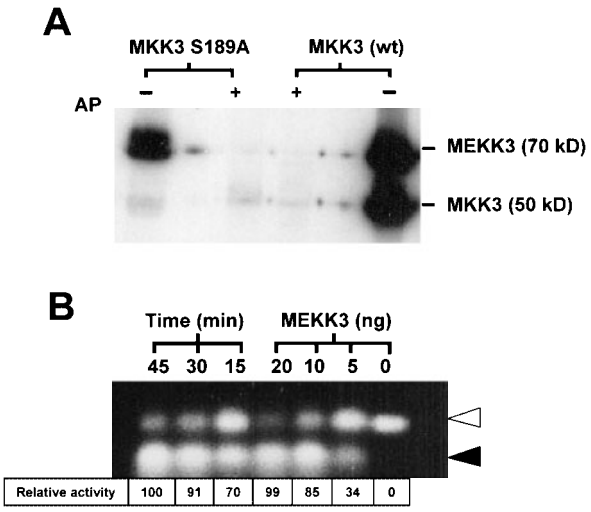

Fig. 2. **Recombinant MEKK3 phosphorylates Ser[189] on MKK3.** To determine whether MEKK3 phosphorylates Ser[189] on MKK3, a protein kinase assay was performed in the presence of [γ-[32]P]ATP with MEKK3 and two recombinant MKK3 proteins, wild type (wt) MKK3 and mutant MKK3 S189A. As a control, alkaline phosphatase (AP) was added to the kinase assay products. The phosphorylated MEKK3 and MKK3 are indicated in (A). To establish an MEKK3 kinase activity assay system, the flourescein-conjugated MKK3 peptide (FITC-[183]SGYLVDSVAKTIA[195]) was used. The amount of phosphorylated MKK3 peptide increased in a time- and MEKK3 concentration dependant manner. The phosphorylated product (solid arrowheads) and the unphosphorylated substrate (open arrowheads) are indicated in (B). The mean relative activity is indicated below.

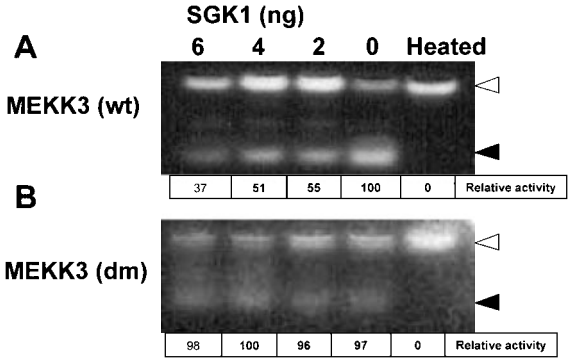

Fig. 3. **Inhibition of the protein kinase activity of MEKK3 by SGK1.** To confirm that the phosphorylation of Ser[166] and Ser[337] on MEKK3 affects the MEKK3 kinase activity, we measured the kinase activity of MEKK3 and the phosphorylation of the serine residues with and without SGK1 pretreatment. Twenty nanograms of immuno-precipitated wt MEKK3 and dm MEKK3 S166A S337A were pretreated with SGK1 (0, 2, 4, and 6 ng for 20 min at 30°C), and the MEKK3 kinase assay was performed with the MKK3 peptide (FITC-[183]SGYLVDSVAKTIA[195]). The kinase activity of wt MEKK was reduced by SGK1 pretreatment (A), while that of dm MEKK3 S166A S337A was not affected (B). The mean relative activity of four independent experiments is indicated below.

type MKK3 was phosphorylated by MEKK3 while the mutant MKK3 S189A was not. The phosphorylated bands disppeared upon treatment with alkaline phosphatase (AP). To clarify the phosphorylation of Ser[189] onMKK3 and to establish an MEKK3 kinase activity assay system, we used a fluorescein-conjugated MKK3 peptide (FITC-[183]SGYLVDSVAKTIDA[195]). As shown in Fig. 2B, the amount of phosphorylated MKK3 peptide increased depending on the concentration of MEKK3 and the incubation time. To verify the specificity of the MKK3 peptide as a substrate for MEKK3, the peptide was incubated with PKC and GSK3 instead of MEKK3. Other fluorescein-labeled peptides including IRS-1, PDK1, and CDC42 were also incubated with MEKK3. However, there was no detectable phosphorylated product in any of these reactions (data not shown). Thus, it was demonstrated that MEKK3 phosphorylates Ser[189] on MKK3, and that this MEKK3 kinase assay system using a fluorescein-conjugated MKK3 peptide is feasible. We used this MEKK3 assay system to monitor changes in the kinase activity of MEKK3 *in vivo* and *in vitro*.

SGK1 Phosphorylates MEKK3 and Inhibits the Kinase Activity of MEKK3 In Vitro and In Vivo—MEKK3 plays various roles in the control of cell shape, adhesion, movement, endocytosis, secretion and downstream SAPK activity (*1–3*, *28*). The kinase activity of MEKK3 was assayed with or without SGK1 pretreatment *in vitro*, in order to investigate whether the function of MEKK3 is affected by its phosphorylation by SGK1. The recombinant wild type MEKK3 and the double mutant MEKK3 S166A S337A were expressed in *E. coli* and purified. The purified proteins were treated with 0–6 ng of active SGK1 and their kinase activities were measured. The kinase activity of wt MEKK3 was reduced depending on the SGK1 concentration (Fig. 3A), while that of dm MEKK3 S166A S337A remained constant regardless of SGK1 pretreatment (Fig. 3B). These results suggest that SGK1 inhibits the protein kinase activity of MEKK3 by phosphorylating MEKK3 *in vitro*.

To confirm this *in vivo*, 1 μg of wt GFP-MEKK3 or dm GFP-MEKK3 S166A S337A was co-transfected with GST-SGK1 into COS-1 cell at different DNA ratios of MEKK3 to SGK1 (0:0, 1:1, 1:2, 1:4, and 1:8). GFP-MEKK3 and GST-SGK1 were immunoprecipitated, respectively, with anti-GFP and the anti-GST antibodies. To monitor the expression level of the transfected MEKK3 and SGK1, Western blot analyses were performed. SGK1 expression increased with the increase in the ratio of MEKK3 to SGK1 (middle lower Fig. 4, A and B), while MEKK3 expression remained constant (bottom in Fig. 4, A and B). In the same samples, the activities of MEKK3 and SGK1 were assayed, respectively, with flourescein-conjugated MKK3 peptide (FITC-[183]SGYLVDSVAKTIA[195]) and MEKK3 peptide (FITC-[159]ep*Rs*R*h*lSVi[168]) as substrates. As shown in Fig. 4A top, the kinase activity of wt MEKK3 declined along with the increase of SGK1 DNA concentration, meanwhile that of dm MEKK3 S166A S337A was unchanged regardless of the increase in SGK1 DNA concentration (Fig. 4B, top). Thus, these results also suggest that SGK1 phosphorylates MEKK3 and inhibits MEKK3 protein kinase activity *in vivo*. Therefore, we demonstrated that the MEKK3 kinase activity on Ser[189] of MKK3, which corresponds to Ser[207] of MKK6, is inhibited by the phosphorylation of MEKK3 by SGK1 both *in vitro* and *in vivo*. Thus, the SGK1 signal transduction pathway seems to be antago-

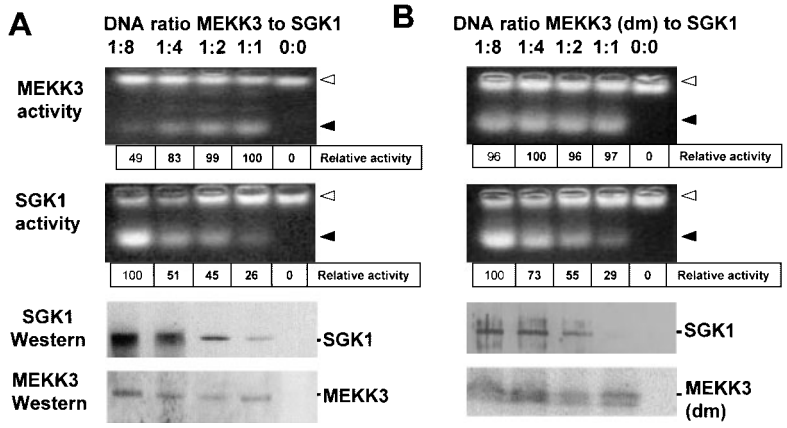

Fig. 4. **SGK1 phosphorylates and inhibits MEKK3 *in vivo*.** To evaluate the effect of the phosphorylation of MEKK3 by SGK1, COS-1 cells were co-transfected with different DNA ratios of wt MEKK3 or dm MEKK3 S166A S337A to SGK1 (0:0, 1:1, 1:2, 1:4, and 1:8). MEKK3 kinase activity (top in A and B) and the SGK1 activity (middle upper in A and B) were measured with the flourescein-conjugated MKK3 peptide and the MEKK3 peptide (FITC-^{159}epRsRhlSVi^{168}), respectively. The phosphorylated products (solid arrowheads) and unphosphorylated substrate (open arrowheads) are marked. Along with the DNA ratio of MEKK3 (wt or dm) to SGK1, both the expression (middle lower in A and B) and activity of SGK1 increased while MEKK3 expression (bottom in A and B) remained unchanged. Western blot analyses for MEKK3 and SGK1 were performed with anti-MEKK3 and anti-SGK1 antibodies. The kinase activity of MEKK3 (wt) in the cells declined in contrast to the increase in the DNA concentration and activity of SGK1 (A), while that of the double mutant remained constant in spite of the increased DNA concentration and activity of SGK1 (B). The mean relative activity of four experiments is indicated below.

nistic to the MKK3/6-SAPK1/2 signal pathway (*25*, *28*, *29*). MEKK3 has diverse functions depending on cell type, so it remains to be determined whether MEKK3 is regulated by SGK1 in other cell lines/organisms.

It has been reported that MEKK3 regulates the SAPK1/2 and NF-κB pathways (*1–4*, *30*). Although we demonstrated that SGK1 regulates MEKK3 and its downstream MKK3/6 activities, it is also important to establish whether SGK1 is involved in the activation of NF-κB in the MEKK3 signal transduction pathway (*30*).

The identification and characterization of the target proteins of SGK1 may provide clues to understanding how SGK1 contributes to the survival and death of cells. We noticed that the apoptosis signal-regulating kinases 1/2 (ASK1/2) contain SGK1/Akt1 phosphorylation motifs. ASK1/2 are involved in apoptosis by activating the SEK1-SAPK1 and MKK3/6-SAPK2 signal cascades (*29*, *31*, *32*). Thus, it is suggested that SGK1 and Akt1 may phosphorylate ASK1/2 and down-regulate them.

MEKK3 is involved in apoptosis and cell growth, so the upstream of MEKK3 signal pathway seems to be related to the bifurcation of the functions of MEKK3. Which extracellular signals regulate MEKK3 remains to be shown. It has been reported that 14–3-3 proteins bind to the amino-terminal regulatory and carboxy-terminal kinase domains of MEKK3 (*33*). This suggests that 14-3-3 proteins are important for the regulation of MEKK3 by mediating interactions between MEKK3 and its regulatory proteins. As demonstrated in this study, SGK1 phosphorylates MEKK3 to inactivate it. So one of these regulatory proteins under the influence of the interaction between 14-3-3 and MEKK3 is thought to be SGK1. We also notice that one of the 14-3-3 binding motifs (R-$X_{1\text{-}2}$-S-$X_{2\text{-}3}$-pS) overlaps both SGK1 phosphorylation sites (Ser^{166} and Ser^{337}) of MEKK3 (34). Thus, we are now investigating crosstalk between SGK1, MEKK3 and 14–3-3.

We demonstrated that SGK1 phosphorylates Ser^{166} and Ser^{337} on MEKK3 by using a recombinant MEKK3 protein and fluorescein-labeled MEKK3 peptides. This phosphorylation leads to the inactivation of MEKK3, resulting in a decrease in the phosphorylation of Ser^{189} on MKK3.

This research was supported by a grant to S S Kang from the Korea Research Foundation (KRF-2000–003-D00132). We thank Dr Alessi, Dr. Schenbelist and Dr. Han for generously providing SGK1, MEKK3 and MKK3 cDNA, respectively, as gifts.

REFERENCES

1. Deacon, K. and Blank, J.L. (1997) Characterization of the mitogen-activated protein kinase kinase 4 (MKK4)/c-Jun NH2-terminal kinase 1 and MKK3/p38 pathways regulated by MEK kinases 2 and 3. MEK kinase 3 activates MKK3 but does not cause activation of p38 kinase *in vivo*. *J. Biol. Chem.* **272**, 14489–14496
2. Deacon, K. and Blank, J.L. (1999) MEK kinase 3 directly activates MKK6 and MKK7, specific activators of the p38 and c-Jun NH2-terminal kinases. *J. Biol. Chem.* **274**, 16604–16610
3. Schlesinger, T.K., Fanger, G.R., Yujiri, T., and Johnson, G.L. (1998) The TAO of MEKK. *Front Biosci.* **3**, 1181–1186
4. Zanke, B.W., Rubie, E.A., Winnett, E., Chan. J., Randall, S., Parsons, M., Boudreau, K., McInnis, M., Yan, M., Templeton, D.J., and Woodgett, J.R. (1996) Mammalian mitogen-activated protein kinase pathways are regulated through formation of specific kinase-activator complexes. *J. Biol. Chem.* **271**, 29876–29881
5. Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J.A., Lin, S., and Han, J. (1996) Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). *J. Biol. Chem.* **271**, 17920–17926
6. Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B., and Davis, R.J. (1997) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell Biol.* **16**, 1247–1255
7. Han, J., Lee, J.D., Jiang, Y., Li, Z., Feng, L., and Ulevitch, R.J. (1996) Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J. Biol. Chem.* **271**, 2886–2891
8. Ellinger-Ziegelbauer, H., Kelly, K., and Siebenlist, U. (1999) Cell cycle arrest and reversion of Ras-induced transformation by a conditionally activated form of mitogen-activated protein kinase kinase kinase 3. *Mol. Cell Biol.* **19**, 3857–3868
9. Webster, M.K., Goya, L., Ge, Y., Maiyar, A.C., and Firestone, G.L. (1993) 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
10. Webster, M.K., Goya, L., and Firestone, G.L. (1993) Immediate-early transcriptional regulation and rapid mRNA turnover

of a putative serine/threonine protein kinase. *J. Biol. Chem.* **268**, 11482–11485
11. 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
12. 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
13. 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
14. Imaizumi, K., Tsuda, M., Wanaka, A., Tohyama, M., and Takagi, T. (1994) Differential expression of sgk m*RNA*, a member of the Ser/Thr protein kinase gene family, in rat brain after CNS injury. *Brain Res. Mol. Brain Res.* **2**, 189–196
15. Webster, M.K., Goya, L., and Firestone, G.L. (1993) Immediate-early transcriptional regulation and rapid mRNA turnover of a putative serine/threonine protein kinase. *J. Biol .Chem.* **268**, 11482–11485
16. Waldegger, S., Gabrysch, S., Barth, P., Fillon, S., and Lang, F. (2000) h-sgk serine-threonine protein kinase as transcriptional target of p38/MAP kinase pathway in HepG2 human hepatoma cells. *Cell Physiol. Biochem.* **10**, 203–208
17. 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
18. 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**, 233–236
19. 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
20. 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
21. Xu, J., Liu, D., Gill, G., and Songyang, Z. (2001) Regulation of cytokine-independent survival kinase (CISK) by the Phox homology domain and phosphoinositides. *J. Cell Biol.* **154**, 699–705
22. Mikosz, C.A, Brickley, D. R, Sharkey, M.S., Moran, T.W., and Conzen, S.D. (2001) Glucocorticoid receptor-mediated protection from apoptosis is associated with induction of the serine/threonine survival kinase gene, sgk-1. *J. Biol. Chem.* **276**, 16649–16654
23. 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
24. Zhang, B.H., Tang, E.D., Zhu, T., Greenberg, M, E., Vojtek, A.B., and Guan, K.L. (2001) Serum- and glucocorticoid-inducible kinase SGK phosphorylates and negatively regulates B-Raf. *J. Biol. Chem.* **276**, 31620–31626
25. Gratton, J.P., Morales-Ruiz, M., Kureishi, Y., Fulton, D., Walsh, K., and Sessa, W.C. (2001) Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor-mediated cytoprotection in endothelial cells. *J. Biol. Chem.* **276**, 30359–30365
26. Kang, S.S., Kwon, T., Kwon, D.Y., and Do, S.I. (1999) Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J. Biol. Chem.* **274**, 13085–13090
27. Kwon, T., Kwon, D.Y., Chun, J., Kim, J.H., and Kang, S.S. (2000) Akt protein kinase inhibits Rac1-GTP binding through phosphorylation at serine 71 of Rac1. *J. Biol. Chem.* **275**, 423–428
28. Wang, Z., Jiang, C., Ganther, H., and Lu, J. (2001) Antimitogenic and proapoptotic activities of methylseleninic acid in vascular endothelial cells and associated effects on PI3K-AKT, ERK, JNK and p38 MAPK signaling. *Cancer Res.* **61**, 7171–7178
29. Park, H.S., Kim, M.S., Huh, S.H., Park, J., Chung, J., Kang, S.S., and Choi E.J. (2002). Akt kinase (protein kinase B) negatively regulates SEK1 by means of protein phosphorylation. *J. Biol. Chem.* **277**, 2573–2578
30. Zhao, Q. and Lee, F.S. (1999) Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase-beta. *J. Biol. Chem.* **274**, 8355–8358
31. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, and K., Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J.* **17**, 2596–25606
32. Fanger, G.R., Widmann, C., Porter, A.C., Sather, S., Johnson, G.L., and Vaillancourt, R.R. (1998) 14–3-3 proteins interact with specific MEK kinases. *J. Biol. Chem.* **273**, 3476–3483
33. van Hemert, M.J., Steensma, H.Y., and van Heusden, G.P.H. (2001) 14–3-3 proteins: key regulators of cell division, signalling and apoptosis. *Bioessays* **23**, 936–946