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Negative regulation of SEK1 signaling by serum- and glucocorticoid-inducible protein kinase 1

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Serum- and glucocorticoid-inducible protein kinase 1 (SGK1) has been implicated in diverse cellular activities including the promotion of cell survival. The molecular mechanism of the role of SGK1 in protection against cellular stress has remained unclear, however. We have now shown that SGK1 inhibits the activation of SEK1 and thereby negatively regulates the JNK signaling pathway. SGK1 was found to physically associate with SEK1 in intact cells. Furthermore, activated SGK1 mediated the phosphorylation of SEK1 on serine 78, resulting in inhibition of the binding of SEK1 to JNK1, as well as to MEKK1. Replacement of serine 78 of SEK1 with alanine abolished SGK1-mediated SEK1 inhibition. Oxidative stress upregulated SGK1 expression, and depletion of SGK1 by RNA interference potentiated the activation of SEK1 induced by oxidative stress in Rat2 fibroblasts. Moreover, such SGK1 depletion prevented the dexamethasone-induced increase in SGK1 expression, as well as the inhibitory effects of dexamethasone on paclitaxel-induced SEK1-JNK signaling and apoptosis in MDA-MB-231 breast cancer cells. Together, our results suggest that SGK1 negatively regulates stress-activated signaling through inhibition of SEK1 function.

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Introduction

Serum- and glucocorticoid-inducible protein kinase 1 (SGK1) is a multifunctional serine–threonine kinase that was originally identified as the product of a gene whose transcription was controlled by serum and glucocorticoids in rat mammary tumor cells (Webster *et al*, 1993a). SGK1 is implicated in a variety of cellular activities including the regulation of ion channel conductance, cell volume, and cell survival (Brunet *et al*, 2001; Mikosz *et al*, 2001; Wang *et al*, 2001; Busjahn *et al*, 2002; Gamper *et al*, 2002; Wulff *et al*, 2002). It belongs to the AGC family of protein kinases, which also includes cAMP-dependent protein kinase (protein kinase A), cGMP-dependent protein kinase (protein kinase G), and protein kinase C. The catalytic domain of SGK is highly similar to those of Akt (protein kinase B), protein kinase A, p70 S6 kinase, and protein kinase C (Webster *et al*, 1993b). Also like Akt, SGK1 is activated by phosphorylation, in response to stimulation of the phosphoinoside 3-kinase (PI3K) signaling pathway by insulin and other growth factors (Lang and Cohen, 2001). SGK1 can be also transcriptionally upregulated by a variety of stimuli including osmotic stress (Bell *et al*, 2000), cortical brain injury (Imaizumi *et al*, 1994), DNA damaging agents (Waldegger *et al*, 2000), TGF-β (Reeves *et al*, 2000), and follicle stimulating hormone (Gonzalez-Robayna *et al*, 1999), in addition to serum (Webster *et al*, 1993b). Activated SGK1 mediates phosphorylation of FKHRL1, a member of the Forkhead family of transcription factors (Brunet *et al*, 2001), as well as of GSK3β (Kobayashi *et al*, 1999), B-Raf (Zhang *et al*, 2001), and Nedd4-2 (Debonneville *et al*, 2001). *In vitro* studies have revealed that SGK1, like Akt, prefers to phosphorylate serine or threonine residues of substrate proteins that are present in the RXRXX(S/T) sequence motif (where X is any amino acid).

Mitogen-activated protein kinase (MAPK) signaling pathways mediate information transfer from extracellular stimuli to the nucleus. Mammalian MAPKs are classified into at least three subgroups: extracellular signal-regulated kinase, c-Jun amino-terminal kinase (JNK; also known as stress-activated protein kinase, or SAPK), and p38 MAPK (Manning and Davis, 2003; Raman and Cobb, 2003). Each MAPK signaling pathway is composed of three kinase components: a MAPK, a MAPK kinase (MAP2K), and a MAPK kinase kinase (MAP3K). When activated, MAP3Ks phosphorylate and thereby activate MAP2Ks, which in turn phosphorylate and activate MAPKs. The JNK pathway consists of JNK, a MAP2K such as SEK1 (also known as MKK4 or JNKK1) or MKK7, and a MAP3K such as MEKK1 or ASK1 (Sanchez *et al*, 1994; Ichijo *et al*, 1997; Moriguchi *et al*, 1997; Rangone *et al*, 2004). This pathway is activated by a variety of cellular stressors, including genotoxic agents, and proinflammatory cytokines such as tumor necrosis factor-alpha (Manning and Davis, 2003).

To clarify the role of SGK1 in intracellular signaling cascades, we now have investigated the effects of this kinase on the JNK/SAPK signaling pathway. We show that SGK1, when activated, prevents stimulation of the JNK/SAPK signaling pathway. SGK1 physically interacts with and phosphorylates SEK1, thereby inhibiting SEK1 activation and downstream

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signaling events. We also found that dexamethasone, which induces SGK1 expression in various cell types (Firestone *et al*, 2003), inhibited the paclitaxel-induced activation of SEK1-JNK1 signaling and apoptosis in breast cancer cells, and that this inhibition was itself blocked by RNA interference (RNAi)-mediated depletion of SGK1.

Results

SGK1 inhibits activation of SEK1 and JNK1 but not that of MEKK1

To investigate the possible effect of SGK1 on the JNK signaling pathway, we first transfected 293T cells with an expression vector for HA-tagged JNK1, alone or together with a vector for a Flag-tagged constitutively active form of SGK1 (SGK1-CA). Exposure of the transfected cells to anisomycin resulted in stimulation of JNK1 activity, and this stimulation was inhibited by SGK1-CA (Figure 1A). We next examined whether SGK1-CA could inhibit the anisomycin-induced activation of SEK1 and MEKK1, which function as a MAP2K and a MAP3K, respectively, of the JNK signaling pathway. Expression of SGK1-CA inhibited the anisomycin-stimulated activity of SEK1 (Figure 1B) but not MEKK1 (Figure 1C). SGK1-CA also inhibited the MEKK-induced activation of SEK1 but not MKK7 (Supplementary Figure 1). These results thus suggested that SGK1-CA blocks activation of the MEKK1-SEK1-JNK signaling pathway by inhibiting SEK1 activity. We also examined the effect of serum-induced activation of SGK1 on SEK1 activity. Exposure of serum-deprived cells to 10% serum resulted in an increase in the kinase activity of SGK1 that was apparent within 15 min (data not shown). Serum-activated SGK1 inhibited the anisomycin-stimulated activity of SEK1 in 293T cells transfected with expression vectors for GST-SEK1 and SGK1-Flag (Figure 1D).

SGK1 binds and phosphorylates SEK1

Given that SEK1 appeared to be a target of SGK1, we investigated whether endogenous SGK1 physically associates with endogenous SEK1 in intact cells. Immunoblot analysis

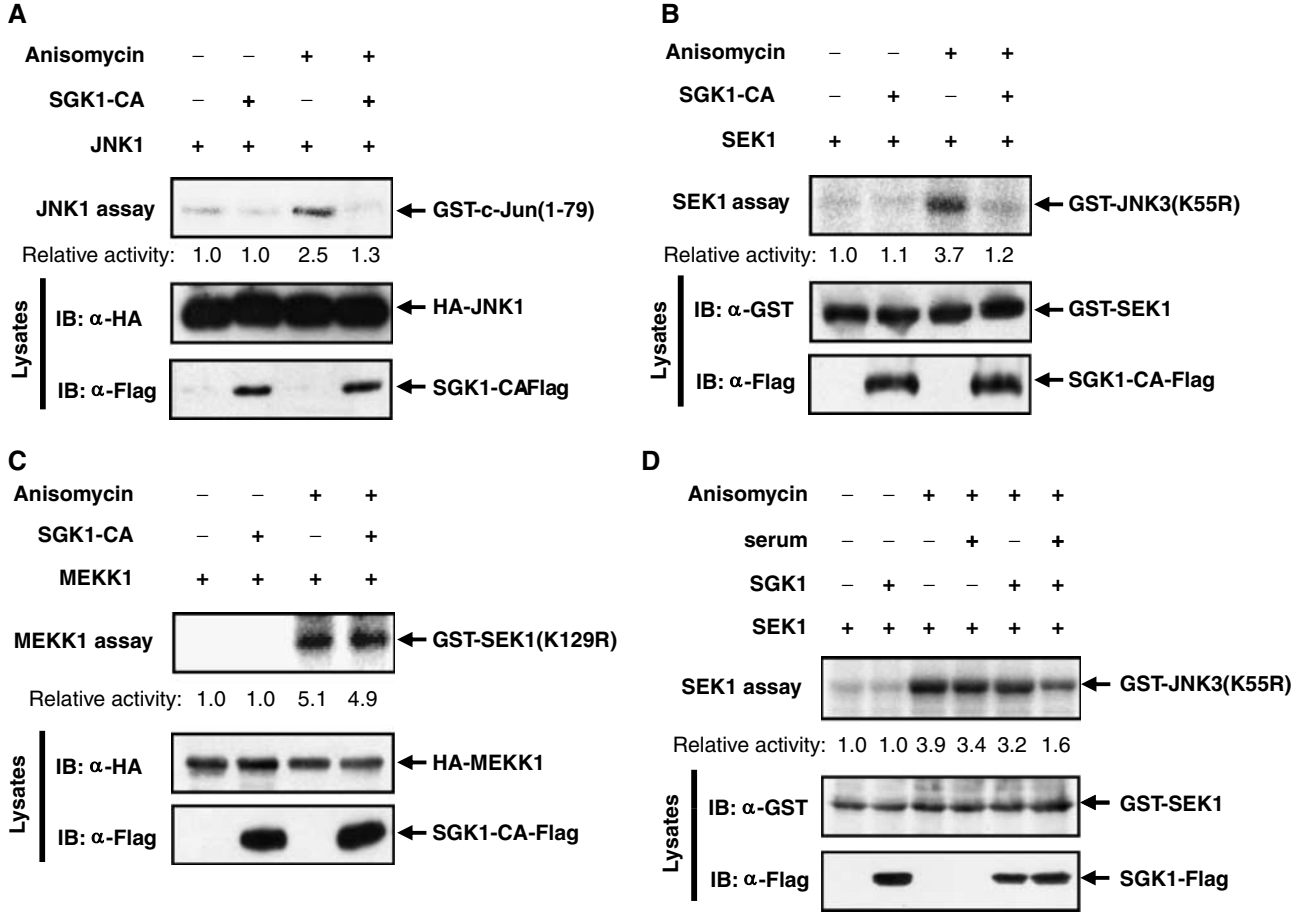

Figure 1 SGK1 inhibits the activation of JNK1 and SEK1, but not MEKK1. (**A–C**) 293T cells were transfected for 48 h with expression vectors for HA-JNK1 (A), GST-SEK1 (B), or HA-MEKK1 (C), either alone or together with a vector for Flag-tagged SGK1-CA. The cells were then incubated for 20 min in the absence or presence of anisomycin (10 μg/ml), after which cell lysates were prepared and subjected to immunoprecipitation with antibodies to HA (A, C) or GST (B). The resulting precipitates were assayed for JNK1 (A), SEK1 (B), or MEKK1 (C) activity by immune complex kinase assays. The amounts of the recombinant proteins in cell lysates were also examined by immunoblot (IB) analysis with the indicated antibodies. (**D**) 293T cells were transfected for 48 h with an expression vector for GST-SEK1, alone or together with a vector for SGK1-Flag. The cells were then deprived of serum for 16 h before incubation first for 20 min in the absence or presence of 10% FBS, and then for 20 min in the absence or presence of 10 μg/ml anisomycin. Cell lysates were subjected to immunoprecipitation with anti-GST antibody, and the resulting precipitates were assayed for SEK1 activity by immune complex kinase assay. Cell lysates were also examined directly by IB analysis with anti-GST and anti-Flag antibodies.

with anti-SEK1 antibody of SGK1 immunoprecipitates prepared from Rat2 fibroblasts revealed that SGK1 indeed physically interacted with SEK1, and that this interaction was not affected by treatment of cells with serum and anisomycin (Figure 2A). In comparison, SGK1 did not physically associate with MKK7, another MAP2K in the JNK signaling pathway (Figure 2B).

Given that SGK1 was found to physically associate with SEK1, we tested whether SGK1 phosphorylates SEK1. We first carried out an *in vitro* phosphorylation assay with a glutathione *S*-transferase (GST) fusion protein of SEK1(K129R), a kinase-inactive mutant of SEK1, as the substrate in order to rule out the possibility of SEK1 autophosphorylation. Immunoprecipitates prepared with anti-Flag antibody from serum-stimulated 293T cells expressing Flag-tagged SGK1 catalyzed the phosphorylation of GST-SEK1(K129R) *in vitro* (Figure 2C), whereas similar immunoprecipitates prepared from serum-stimulated cells expressing a Flag-tagged dominant-negative mutant of SGK1 (SGK1-DN) did not (Figure 2C). These data thus indicated that SGK1 phosphorylates SEK1 *in vitro*. SGK1-Flag immunoprecipitates prepared from serum-treated cells did not phosphorylate GST, GST-c-Jun(1-79), GST-JNK3(K55R), or GST-MKK7 (Figure 2D).

SGK phosphorylates substrate proteins preferentially on serine and threonine residues within the motif Arg-X-Arg-X-X-Ser/Thr (Kobayashi *et al*, 1999). This motif is present at amino acids 73–78 of mouse SEK1 (or amino acids 75–80 of human SEK1). We therefore tested whether SGK1 phosphorylates mouse SEK1 on Ser^{78}. SGK1-CA-Flag immunoprecipitates prepared from transfected 293T cells phosphorylated recombinant GST-SEK1(K129R) *in vitro* (Figure 3A). Substitution of alanine for Ser^{78} of SEK1, however, abolished the *in vitro* phosphorylation of the recombinant protein by SGK1-CA-Flag. We next examined SGK1-mediated phosphorylation of SEK1 in 293T cells by metabolic labeling with

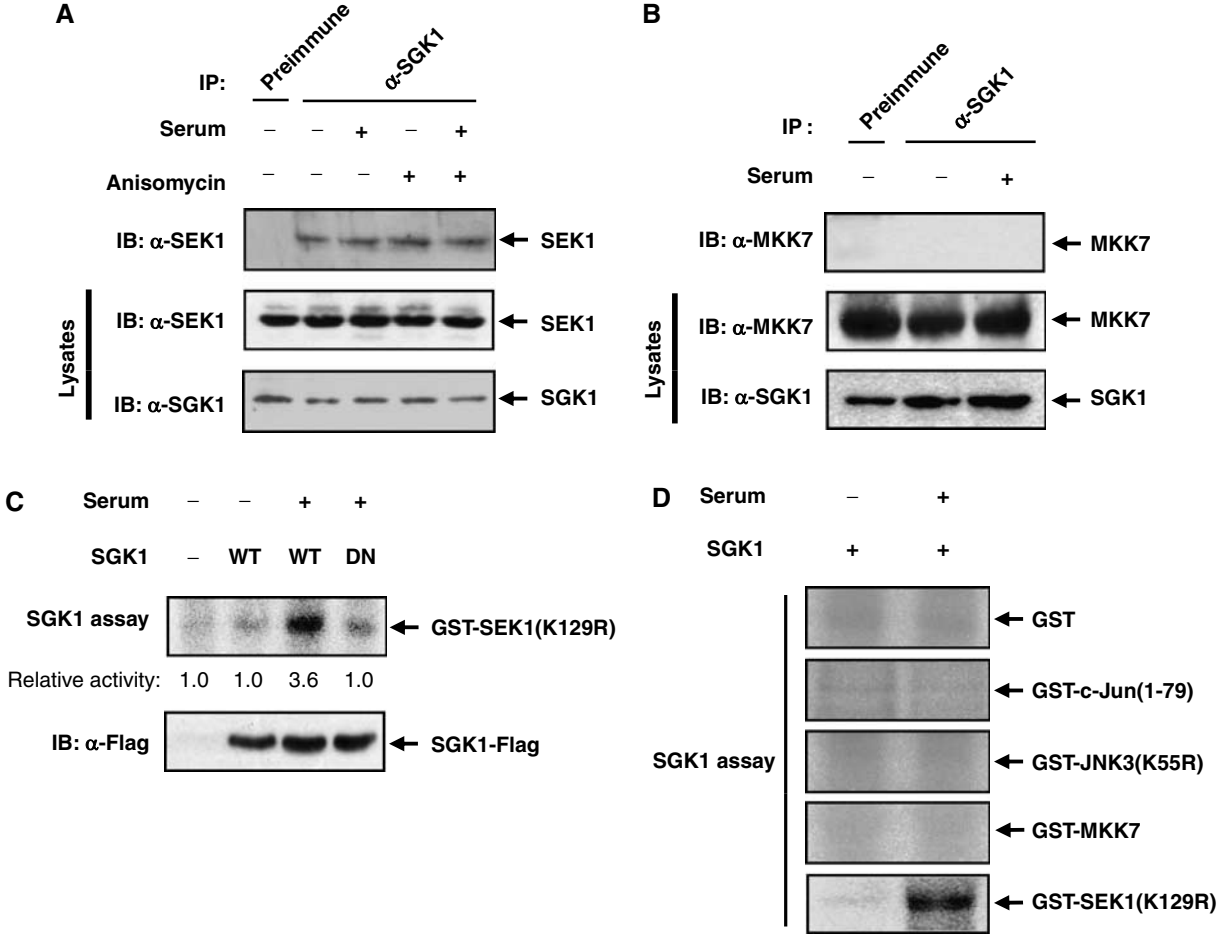

Figure 2 SGK1 physically associates with SEK1. (**A**) Rat2 cells were deprived of serum for 16 h, incubated for 20 min in the absence or presence of 10% FBS, and then incubated further for 20 min in the absence or the presence of 10 μg/ml anisomycin. Cell lysates were then subjected to immunoprecipitation (IP) with anti-SGK1 or rabbit preimmune IgG, and the resulting precipitates were subjected to immunoblot analysis with anti-SEK1 antibody. Cell lysates were also examined directly by immunoblot analysis with antibodies to SGK1 or to SEK1. (**B**) Rat2 cells were deprived of serum for 16 h and then incubated for 20 min in the absence or presence of 10% FBS. Cell lysates were immunoprecipitated with anti-SGK1 or rabbit preimmune IgG, and the resulting precipitates were subjected to immunoblot analysis with anti-MKK7 antibody. Cell lysates were also examined directly by immunoblot analysis with antibodies to SGK1 or to MKK7. (**C, D**) 293T cells were transfected for 48 h with expression vectors for Flag-tagged wild-type (WT) SGK1 or SGK1-DN (C), or with a vector for wild-type SGK1-Flag (D). The cells were then deprived of serum for 16 h before incubation for 20 min in the absence or presence of 10% FBS. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody, and the resulting precipitates were assayed for kinase activity to phosphorylate GST-SEK1 (K129R) (C) or the indicated GST-fusion proteins (D) as substrate.

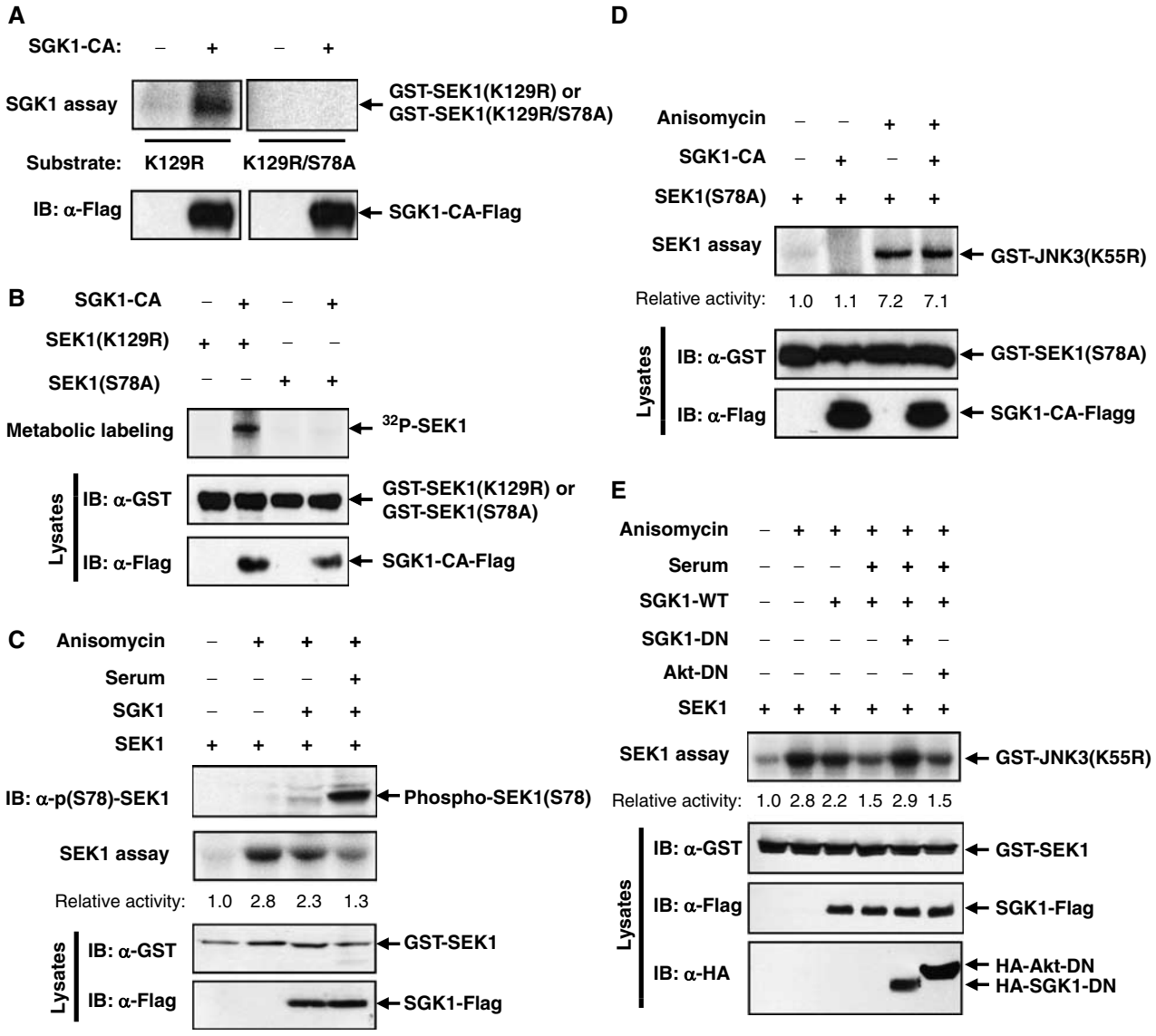

Figure 3 SGK1 phosphorylates SEK1 on serine-78. (**A**) 293T cells were transfected for 48 h with a vector for Flag-tagged SGK1-CA, and cell lysates were then subjected to immunoprecipitation with anti-Flag antibody. The resulting precipitates were examined for kinase activity with GST-SEK1(K129R) or GST-SEK1(K129R/S78A) as the substrate. Cell lysates were also examined directly by immunoblot analysis with anti-Flag antibody. (**B**) 293T cells were transfected for 48 h with the indicated combinations of expression vectors for SGK1-CA-Flag, GST-SEK1(K129R), and GST-SEK1(S78A). The transfected cells were metabolically labeled for 3 h with [^{32}P]orthophosphate (100 μCi/ml), after which cell lysates were subjected to immunoprecipitation with anti-GST antibody. The resulting precipitates were analyzed by SDS–PAGE and autoradiography. Cell lysates were also examined directly by immunoblot analysis with antibodies to Flag or to GST. (**C**) 293T cells were transfected with an expression vector for GST-SEK1, alone or together with a vector for SGK1-Flag. After 48 h of transfection, the cells were deprived of serum for 16 h, and incubated first for 20 min in the absence or presence of 10% FBS and then for 20 min in the absence or presence of 10 μg/ml anisomycin. Cell lysates were subjected to immunoprecipitation with anti-GST antibody, and the resulting precipitates were assayed for SEK1 activity by immune complex kinase assay. Cell lysates were also examined directly by immunoblot analysis with antibodies to phospho-SEK1 (Ser78), GST, or Flag. (**D**) 293T cells were transfected for 48 h with an expression vector for GST-SEK1(S78A), alone or together with a vector for SGK1-CA-Flag. The cells were then incubated for 20 min in the absence or presence of anisomycin (10 μg/ml). Cell lysates were subjected to immunoprecipitation with anti-GST antibody, and the resulting precipitates were assayed for SEK1 activity by immune complex kinase assay. Cell lysates were also examined directly by immmunoblot analysis with antibodies to GST or to Flag. (**E**) 293T cells were transfected for 48 h with an expression vector for GST-SEK1, alone or together with vectors for SGK1-Flag and an HA-tagged dominant-negative mutant of either SGK1 (SGK1-KD) or Akt (Akt-DN), as indicated. The cells were deprived of serum for 16 h, and then incubated for 20 min in the absence or presence of 10% FBS. Cells were then left unexposed or exposed to 10 μg/ml anisomycin for 20 min. Cell lysates were assayed for SEK1 activity as for panel C. The cell lysates were also examined directly by immunoblot analysis with antibodies to GST, to Flag, or to HA.

[^{32}P]orthophosphate after transfection with expression vectors for SGK1-CA-Flag and either GST-SEK1(K129R) or GST-SEK1(S78A). Expression of SGK1-CA induced phosphorylation of GST-SEK1(K129R) but not GST-SEK1(S78A) in the transfected cells (Figure 3B). Next, using phospho-SEK1 (Ser78) antibody, we examined whether serum-activated SGK1 could increase the phosphorylation of SEK1 at Ser^{78} in 293T cells transfected with expression vectors for GST-SEK1 and SGK1-Flag. Immunoblot analysis with phospho-SEK1 (Ser78) antibody and the kinase assay for

SEK1 activity revealed that serum treatment induced the phosphorylation of SEK1 at Ser^{78} in the transfected cells with inhibiting anisomycin-induced SEK1 activation (Figure 3C). We next examined whether the S78A mutation of SEK1 affected the inhibition of SEK1 activity by SGK1. Anisomycin stimulated the kinase activity of GST-SEK1(S78A) in the transfected 293T cells, and this effect was not inhibited by expression of SGK1-CA (Figure 3D). Taken together, these results suggested that Ser^{78} of SEK1 is a critical residue for the inhibitory action of SGK1 on SEK1.

Akt also inhibits SEK1 activity by mediating the phosphorylation of SEK1 on Ser^{78} (Park *et al*, 2002). We therefore examined whether Akt might be involved in the mechanism of SEK1 inhibition by SGK1. The inhibition of the anisomycin-stimulated activity of SEK1 by serum-activated SGK1 in transfected 293T cells was not reversed by expression of a dominant-negative mutant of Akt (Akt-DN) (Figure 3E), or by treatment of the cells with the Akt inhibitor 1L-6-hydroxy-methyl-chiro-inositol 2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (data not shown). In comparison, SGK1-DN reversed the inhibitory effect of serum-activated SGK1 on anisomycin-stimulated SEK1 activity.

We next examined whether SGK1-mediated SEK1 phosphorylation might modulate the binding of SEK1 to JNK. Co-precipitation analysis with 293T cells transiently expressing GST-SEK1 and Myc epitope-tagged JNK1 showed that the physical interaction between these two ectopic proteins was indeed inhibited by coexpression of SGK1-CA (Figure 4A). In contrast, SGK1-CA did not block the interaction between SEK1(S78A) and JNK1 in transfected cells (Figure 4B). Next, in order to test a possible effect of SGK1-mediated SEK1 phosphorylation on the interaction between MEKK1 and SEK1, we performed similar co-precipitation analysis with 293T cells expressing HA-MEKK1, and either GST-SEK1 or GST-SEK1(S78A). Our data revealed that coexpressed SGK1-CA blocked the binding of MEKK1 to SEK1 (Figure 4C), but not SEK1(S78A) (Figure 4D). These results thus suggested that SGK1-mediated phosphorylation of SEK1 on Ser^{78} inhibits the interaction between SEK1 and its substrate, JNK, as well as the interaction between SEK1 and its upstream kinase, MEKK1.

Depletion of SGK1 expression potentiates SEK1 activation induced by H_2O_2

Various cellular stresses, including oxidative, stress induce expression of SGK1 (Leong *et al*, 2003). Indeed, exposure of Rat2 fibroblasts to H_2O_2 resulted in an increase in the cellular levels of SGK1 protein (data not shown). To examine the role of endogenous SGK1 in the regulation of SEK1-JNK signaling, we transfected rat fibroblast Rat2 cells with a vector for

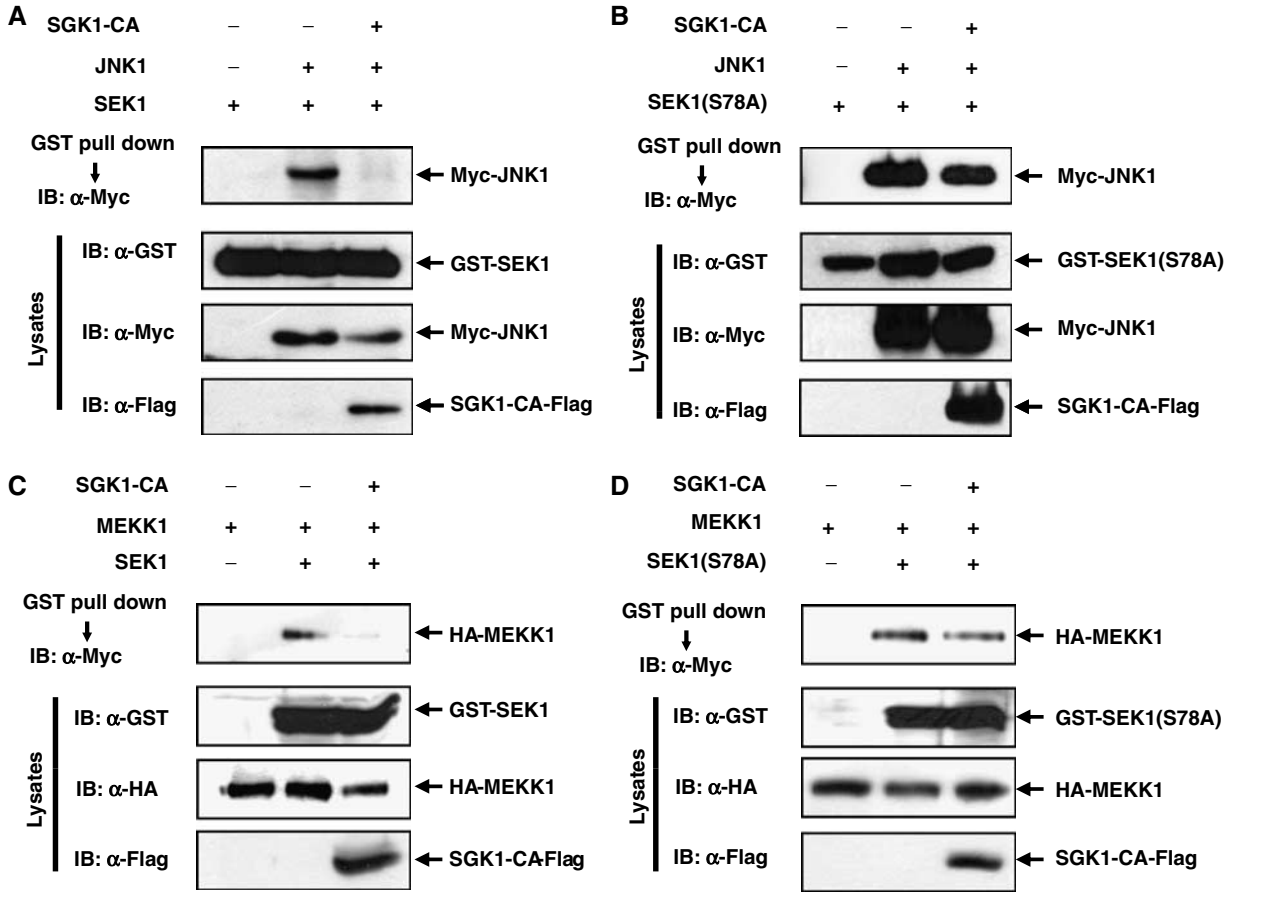

Figure 4 SGK1-mediated phosphorylation of SEK1 inhibits SEK1 binding to JNK as well as to MEKK1. 293T cells were transfected for 48 h with the indicated combinations of plasmid vectors for SGK1-CA-Flag, JNK1-Myc, HA-MEKK1, and either GST-SEK1 (**A, C**) or GST-SEK1(S78A) (**B, D**). The GST fusion proteins were then precipitated from cell lysates with glutathione–agarose beads, and the precipitates were subjected to immunoblot analysis with anti-Myc antibody (A, B) or anti-HA antibody (C, D). Cell lysates were also examined directly by immunoblot analysis with antibodies to GST, Myc, Flag, or HA.

control GFP or rSGK1 small interfering RNAs (siRNAs). Immunoblot analysis and the immune complex kinase assay with anti-SGK1 antibody revealed that 1-h treatment with 1 mM H_2O_2 increased both the abundance and kinase activity of SGK1 in cells expressing GFP siRNA, and that these effects were blocked in cells expressing rSGK1 siRNA (Figure 5A). Treatment with H_2O_2 also induced activation of SEK1 and JNK1 in the control cells, and these effects were enhanced in cells expressing rSGK1 siRNA. H_2O_2 treatment also increased Ser^{78} phosphorylation of SEK1 in the control cells, but not in cells expressing rSGK1 siRNA (Figure 5B). Collectively, these results thus

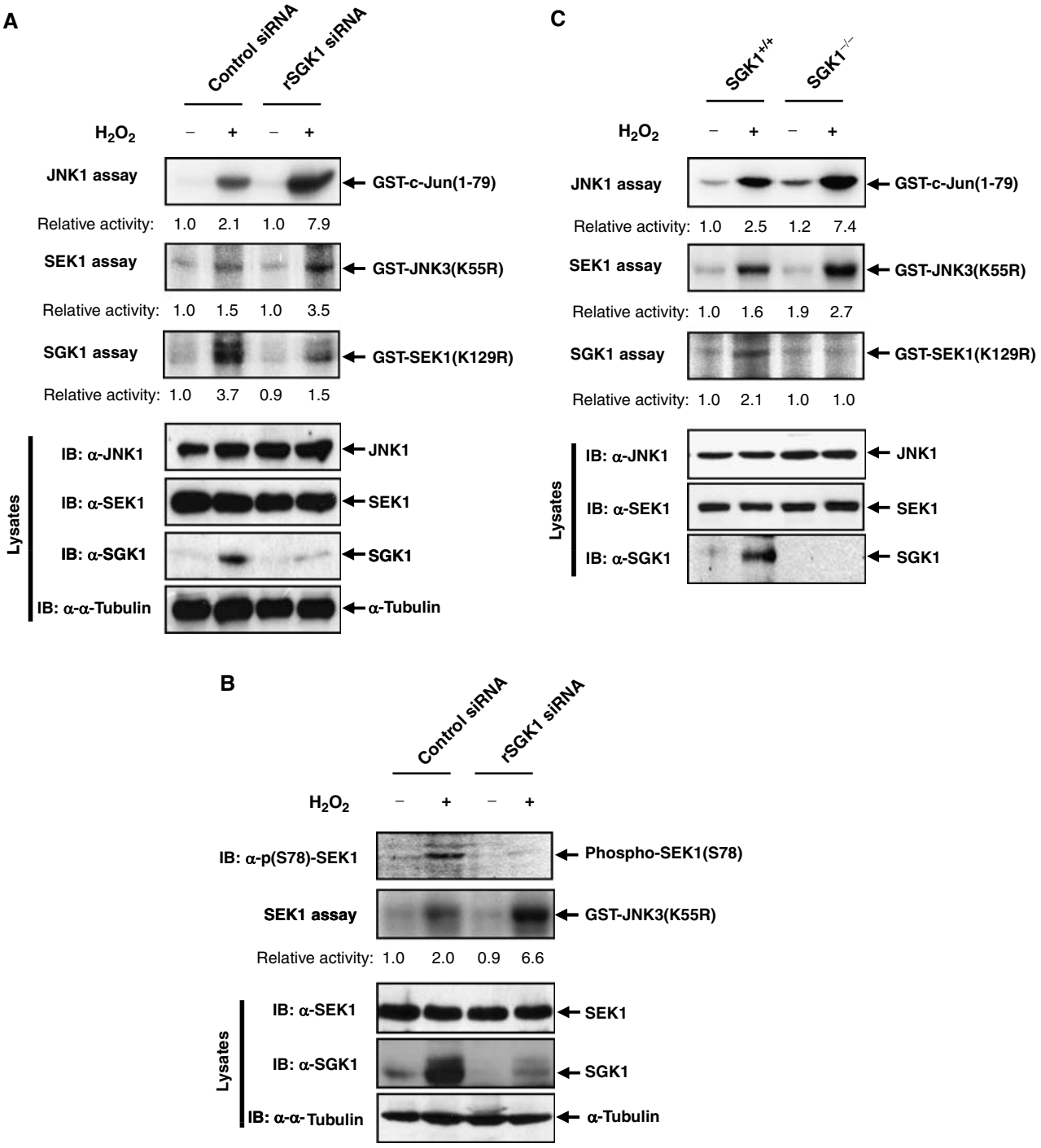

Figure 5 Depletion of SGK1 expression enhances the kinase activities of SEK1 and JNK1 induced by H_2O_2. (**A**, **B**) Rat fibroblast Rat2 cells expressing either control siRNA or rSGK1 siRNA were left untreated or treated with 1 mM H_2O_2 for 1 h. Cell lysates were then subjected to immunoprecipitation with antibodies to SEK1, JNK1, or SGK1 as indicated, and the resulting precipitates were examined for each kinase activity by immune complex kinase assay. Cell lysates were also examined directly by immunoblot analysis with antibodies to SEK1, JNK1, SGK1, phospho-SEK1 (Ser78), or α-tubulin, as indicated. (**C**) MEF cells from $SGK1^{+/+}$ and $SGK1^{-/-}$ mice were left untreated or treated with 1 mM H_2O_2 for 1 h. Cell lysates were then subjected to immunoprecipitation with appropriate antibodies and the precipitates were examined for SEK1, JNK1, and SGK1 activities by immune complex kinase assay as in (A).

suggest that depletion of SGK1 by RNAi potentiated the activation of SEK1 by H_2O_2. We also examined the H_2O_2-stimulated activities of SEK1 and JNK1 in mouse embryonic fibroblast (MEF) cells from $SGK1^{+/+}$ and $SGK1^{-/-}$ mice. H_2O_2 treatment increased both the expression and kinase activity of SGK1 in $MEF^{SGK1(+/+)}$ cells but not in $MEF^{SGK1(-/-)}$ cells (Figure 5C). Under these conditions, the H_2O_2-stimulated activities of SEK1 and JNK1 were higher in $MEF^{SGK1(-/-)}$ cells than $MEF^{SGK1(+/+)}$ cells.

SGK1 depletion by RNAi abolishes dexamethasone-induced inhibition of SEK1-JNK signaling and cell death elicited by paclitaxel in breast cancer cells

Glucocorticoid receptor activation induces SGK1 expression in a variety of cell types, including breast epithelial cells (Tessier and Woodgett, 2006), and the induction of SGK1 expression by glucocorticoids is thought to contribute to the glucocorticoid-induced resistance of breast cancer cells to chemotherapy-induced apoptosis (Wu *et al*, 2004). To investigate whether inhibition of SEK1 by SGK1 underlies such glucocorticoid-induced inhibition of apoptosis, we first examined the effect of dexamethasone on the JNK pathway induced by the chemotherapeutic drug paclitaxel in human MDA-MB-231 breast cancer cells. Paclitaxel stimulated the activities of MEKK1, SEK1, and JNK1 in these cells (Figure 6A). Pretreatment of the cells with dexamethasone resulted in induction of SGK1 expression as well as inhibition of paclitaxel-induced activation of both SEK1 and JNK1 but not MEKK1. Furthermore, immunoblot analysis with phospho-SEK1 (Ser78) antibody revealed that dexamethasone pretreatment increased phosphorylation of SEK1 on Ser^{78} (Figure 6B). These results suggested that dexamethasone inhibits the paclitaxel-induced stimulation of the JNK pathway by blocking SEK1 activation.

To investigate the biological significance of SGK1-mediated SEK1 inhibition in the suppressive effect of dexamethasone on the JNK pathway, we established MDA-MB-231 cell lines expressing one of two hSGK1 siRNAs. Dexamethasone increased both the abundance and kinase activity of SGK1 in MDA-MB-231 cells expressing a control (GFP) siRNA, but not in those expressing hSGK1 siRNA1 or siRNA2 (Figure 7A). Thus, depletion of SGK1 expression by RNAi abolished the dexamethasone-dependent induction of SGK1 in MDA-MB-231 cells. We next investigated the effect of SGK1 depletion on the inhibition by dexamethasone of the paclitaxel-induced activation of the JNK pathway and apoptosis. Whereas exposure of control cells to paclitaxel resulted in stimulation of SEK1 and JNK1 activities in a manner sensitive to pretreatment with dexamethasone, dexamethasone did not inhibit the paclitaxel-induced activation of SEK1 and JNK1 in cells expressing hSGK1 siRNA (Figure 7B). These results thus suggested that SGK1 induced by dexamethasone mediates the inhibitory effects of dexamethasone on the paclitaxel-induced activation of SEK1 and JNK1 in MDA-MB-231 cells.

Paclitaxel induced apoptotic cell death in MDA-MB-231 cells, and this effect was inhibited by expression of a dominant-negative mutant of SEK1 [SEK1(K129R)] (Figure 7C), indicating that SEK1 is involved in the mechanism of paclitaxel-induced apoptosis in these cells. We then examined the effect of dexamethasone on paclitaxel-induced apoptosis, in control or SGK1-depleted MDA-MB-231 cells by

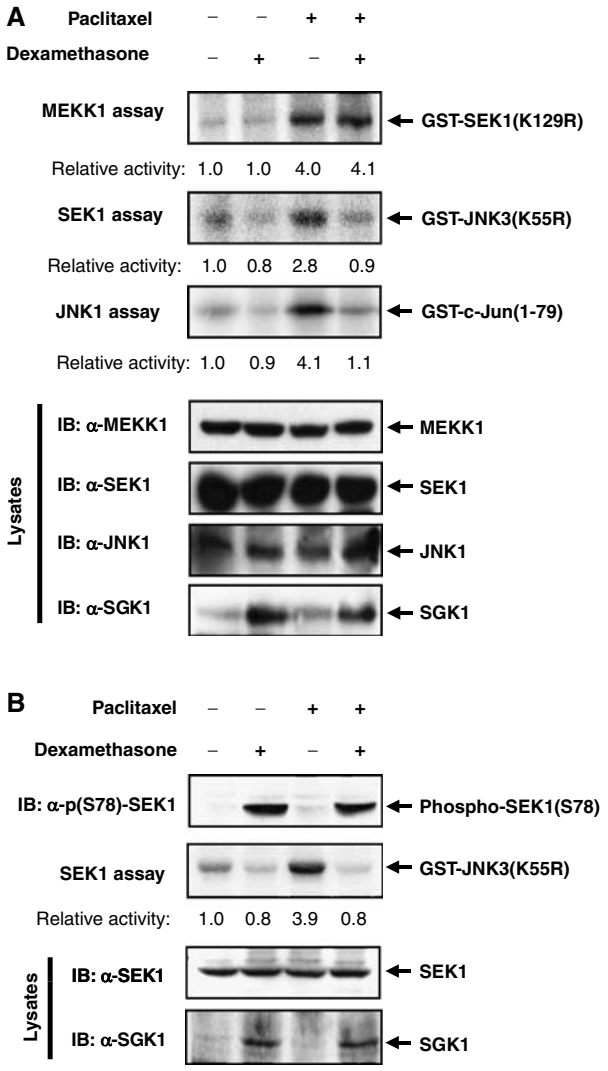

Figure 6 Dexamethasone inhibits paclitaxel-induced SEK1 activation in MDA-MB-231 cells. MDA-MB-231 cells were incubated first in the absence or presence of 1 μM dexamethasone for 2 h, and then incubated further in the absence or presence of 1 nM paclitaxel for 1 h. (**A**) Cell lysates were subjected to immunoprecipitation with antibodies to MEKK1, SEK1, or JNK1, and the resulting precipitates were examined for the respective kinase activities by immune complex kinase assay. Cell lysates were also examined directly by immunoblot analysis with antibodies to MEKK1, SEK1, JNK1, or SGK1. (**B**) Cell lysates were subjected to immunoprecipitation with anti-SEK1 antibody, and the precipitates were assayed for SEK1 activity as in (A). Cell lysates were also examined by immunoblot analysis with antibodies to phospho-SEK1 (Ser78), SEK1, or SGK1.

4′,6-diamidino-2-phenylindole (DAPI) staining (Figure 7D) or TUNEL assay (Figure 7E). Dexamethasone pretreatment inhibited paclitaxel-induced apoptosis in control cells, but not in cells expressing hSGK1 siRNA. These results suggested that SGK1 mediates the dexamethasone-induced resistance of MDA-MB-231 cells to SEK1-dependent apoptosis. Finally, clonal growth assays revealed that paclitaxel blocked colony formation in a manner sensitive to dexamethasone pretreatment in control MDA-MB-231 cells, whereas dexamethasone failed to prevent paclitaxel-induced cytotoxicity in cells expressing hSGK1 siRNA (Figure 7F).

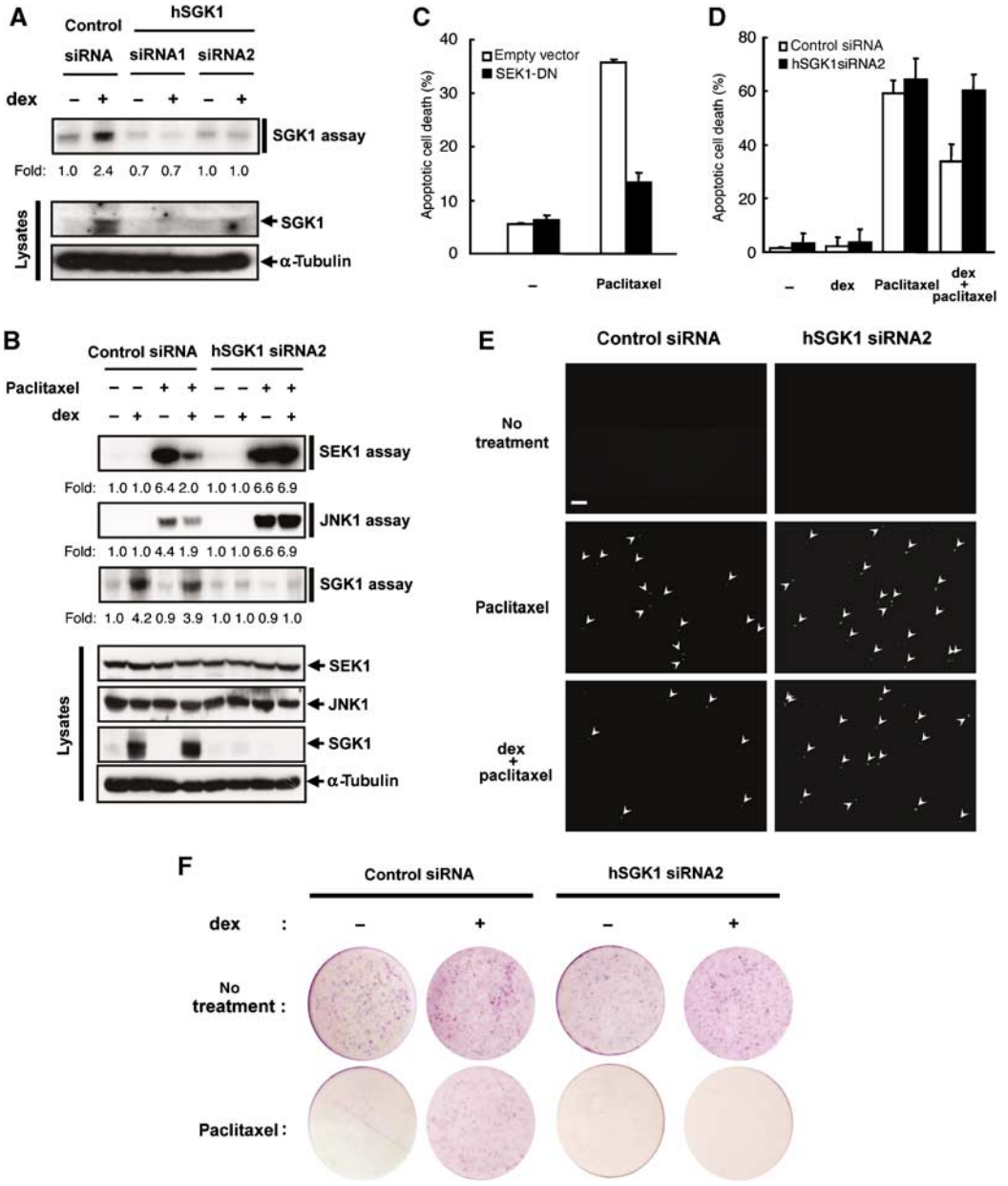

Figure 7 SGK1 depletion by RNAi abolishes the inhibitory effects of dexamethasone on paclitaxel-induced SEK1 activation and cytotoxicity in MDA-MB-231 cells. (**A, B**) MDA-MB-231 cells expressing a control (GFP) siRNA or hSGK1 siRNA1 or siRNA2 were incubated first for 2 h without or with 1 μM dexamethasone (dex), and then for 1 h in the absence or presence of 1 nM paclitaxel, as indicated. In (A), cell lysates were subjected to immunoprecipitation with anti-SGK1 antibody, and the resulting precipitates were examined for SGK1 activity by immune complex kinase assay with GST-SEK1(K129R) as substrate. In (B), cell lysates were subjected to immunoprecipitation with antibodies to SGK1, SEK1, or JNK1, and the resulting precipitates were assayed for the respective kinase activities. Cell lysates were also examined directly by immunoblot analysis, as indicated. (**C**) MDA-MB-231 cells were transfected for 40 h with a vector for GFP, and either a vector for SEK1(K129R) (SEK1-DN) or the corresponding empty vector. After incubation first for 1 h in the absence or presence of 1 nM paclitaxel and then for 24 h in fresh culture medium, the cells were fixed and stained with DAPI. GFP-positive cells were scored for apoptotic nuclei using a fluorescence microscope. Data are means ± s.d. of values from three independent experiments. (**D, E**) MDA-MB-231 cells expressing a GFP control siRNA or hSGK1 siRNA2 were pretreated for 2 h with 1 μM dex, where indicated, and incubated for 1 h in the absence or presence of 1 nM paclitaxel, and then for 24 h in fresh culture medium. They were then fixed and examined for apoptotic nuclear morphology by DAPI staining (D) or by TUNEL assay (E). Data in D are means ± s.d. of values from three independent experiments. Arrowheads in E indicate apoptotic nuclei. Scale bar, 200 μm. (**F**) MDA-MB-231 cells expressing a control siRNA or hSGK1 siRNA2 were pretreated for 1 h with 1 μM dex, where indicated, and incubated for 1 h in the absence or presence of 1 nM paclitaxel. Then, cells were incubated further for 10 days, fixed with 3.5% paraformaldehyde, and stained with 0.5% crystal violet.

Discussion

We have shown that activated SGK1 negatively regulates the JNK signaling pathway by targeting SEK1. SGK1 not only physically interacts with SEK1 but also phosphorylates it both *in vitro* and in intact cells. Amino acids 73–78 of mouse SEK1 match the Arg-X-Arg-X-X-Ser/Thr motif that is preferentially phosphorylated by SGK1 (Kobayashi *et al*, 1999). Indeed, site-specific mutagenesis revealed that SGK1 mediates phosphorylation of SEK1 on Ser^{78}, and that this phosphorylation reaction is required for inhibition of SEK1 by SGK1. Phosphorylation of this residue also abrogated the binding

of SEK1 to MEKK1, as well as to JNK1. The inhibition of SEK1 by SGK1 thus likely results from the SGK1-mediated phosphorylation of SEK1 on Ser^{78}, and consequent inhibition of its interaction with MEKK1 as well as with JNK.

We previously showed that Akt also inhibits the stimulation of SEK1 by mediating its phosphorylation on Ser^{78} (Park *et al*, 2002). Thus, both SGK1 and Akt appear to exert the same effect on SEK1 in the same manner. Given that both SGK1 and Akt are activated by the PI3K-dependent signaling pathway triggered by insulin and other survival factors, the effects of SGK1 and Akt on SEK1 activity might appear to be redundant. However, several lines of evidence suggest that the regulation of SGK1 and Akt is also mediated by distinct mechanisms (Tessier and Woodgett, 2006). For instance, many of cellular stresses such as oxidative stress, osmotic stress, and heat shock, which do not activate Akt, induce transcription of the SGK1 gene and increase SGK1 activity (Bell *et al*, 2000; Leong *et al*, 2003). SGK1 may thus mediate inhibition of the SEK1-JNK signaling axis in response to these diverse stimuli. Many of these cellular stresses also activate SEK1-JNK signaling, however. Indeed, we have shown that H_2O_2-induced oxidative stress triggered activation of SEK1-JNK signaling, as well as increased the abundance and activity of SGK1, in both Rat2 and MEF cells. Upregulation of SGK1 by oxidative stress may therefore reflect an adaptive cellular response to control the stress-induced activation of SEK1-JNK signaling.

Glucocorticoids such as dexamethasone have been administered to suppress nausea and emesis induced by cancer chemotherapy (The Italian Group for Antiemetic Research, 1995, 2000; Kirkbride *et al*, 2000). However, recent studies have shown that glucocorticoids can inhibit chemotherapy-induced apoptosis in a variety of tumor cell types, including breast carcinoma cells (Huang *et al*, 2000; Herr *et al*, 2003; Wu *et al*, 2004). The antiapoptotic properties of glucocorticoids are thought to result from transcriptional stimulation of genes encoding pro-survival proteins including SGK1 (Wu *et al*, 2004). However, the molecular mechanism by which SGK1 mediates the antiapoptotic effects of glucocorticoids has remained unclear. Our results now indicate that inhibition of SEK1 activation by SGK1 contributes to the cytoprotective effect of glucocorticoids in breast cancer cells treated with a chemotherapeutic drug. Given that SGK1 expression levels are high in many breast cancers (Sahoo *et al*, 2005; Zhang *et al*, 2005), inhibition of SGK1-mediated SEK1 phosphorylation may represent a potential therapeutic strategy for improving the effectiveness of antitumor drugs in such cancer cells.

Materials and methods

DNA constructs and antibodies

Complementary DNAs for constitutively active [SGK1(S422D) or SGK1-CA] and dominant-negative [SGK1(K127Q) or SGK1-DN] forms of SGK1 were kindly provided by M Greenberg (Harvard Medical School). Wild-type SGK1, SGK1-CA, and SGK1-DN cDNAs were amplified by the polymerase chain reaction (PCR) and subcloned into the *Eco*RI and *Kpn*I sites of the p3xFlag-CMV-10 vector (Sigma). Vector constructs for the kinase components of the JNK pathway were described previously (Shim *et al*, 1996, 2000; Kim *et al*, 2001; Park *et al*, 2001, 2002). Rabbit polyclonal antibodies to SGK1 and mouse monoclonal antibodies to the Myc epitope were obtained from Cell signaling Technology; mouse monoclonal anti-SEK1 was from BD Pharmingen; mouse polyclonal anti-tubulin and mouse monoclonal antibodies to the hemagglutinin (HA) epitope or Flag epitope tags were from Sigma; rabbit polyclonal phospho-SEK1/MKK4 (S78) antibody, which detects phosphorylation of serine 78 of mouse SEK1 (or serine 80 of human SEK1), was from Abcam Inc.; and mouse monoclonal antibodies to GST and rabbit polyclonal antibodies to MEKK1, MKK7, or JNK1 were from Santa Cruz Biotechnology.

Cell culture and transfection

Human embryonic kidney 293T, rat fibroblast Rat2, human breast cancer MDA-MB-231, and MEF cells from wild-type or $SGK1^{-/-}$ mice (Wulff *et al*, 2002) were maintained in a humidified atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin (100 U/ml)–streptomycin (100 μg/ml) (Hyclone). Cells were transfected by the calcium phosphate method or with Lipofectamine (Invitrogen).

RNAi

Rat2 and MDA-MB-231 cells were stably transfected with pSuper-retro vector encoding either a SGK1 siRNA or a control (GFP) siRNA (Hamar *et al*, 2004). Stable transfectants of Rat2 and MDA-MB-231 cells were selected in the presence of puromycin (1 and 0.2 μg/ml, respectively). The DNA sequences corresponding to the siRNAs were generated and inserted into the *Bgl*II and *Hin*dIII sites of pSuper-retro, as described previously (Brummelkamp *et al*, 2002). The sequences of the PCR primers for rat SGK1 (rSGK1) siRNA were 5′-gatccccCATCGAGCACAATGGGACAttcaagagaTGTCCCATTGTGCTCG ATGtttttggaaa-3′ (forward) and 5′-agcttttccaaaaaCATCGAGCACAATGG GACAtctcttgaaTGTCCCATTGTGCTCGATGggg-3′ (reverse), those for human SGK1 (hSGK1) siRNA1 were 5′-gatccccCAATTCTCATCGCTTT CATGAttcaagagaTCATGAAAGCGATGAGAATTGtttttggaaa-3′ (forward) and 5′-agcttttccaaaaaCAATTCTCATCGCTTTCATGAtctcttgaaTCATGAA AGCGATGAGAATTGggg-3′ (reverse), and those for hSGK1 siRNA2 were 5′-gatccccGTCCTTCTCAGCAAATCAACCttcaagagaGGTTGATTTG CTGAGAAGGACtttttggaaa-3′ (forward) and 5′-agcttttccaaaaaGTCCTTC TCAGCAAATCAACCtctcttgaaGGTTGATTTGCTGAGAAGGACggg-3′ (reverse); the sequences corresponding to the RNA duplexes are shown in uppercase.

Apoptotic cell death

MDA-MB-231 cells expressing either control siRNA or SGK1 siRNA were incubated with 1 μM dexamethasone or 1 nM paclitaxel, as indicated, fixed with 4% paraformaldehyde, and stained with DAPI (10 μg/ml). DAPI-stained nuclei of cells were then examined for apoptotic morphology by fluorescence microscopy, and the percentage of apoptotic cells was determined. Alternatively, cells were analyzed for apoptosis by TUNEL assay with *in situ* cell death detection kit (Roche Applied Science). Images in TUNEL assay were analyzed by using a CCD camera (AxioCam HRc; Carl Zeiss Microimaging Inc.) affixed to a Zeiss Axiovert 200 fluorescence microscope (inverted type, ×200, 0.25 objective lenses) and Axiovision software version 3.1. Image was postprocessed using Photoshop CS 8.0.

Co-immunoprecipitation

Cells were lysed in buffer A (20 mM Tris–HCl (pH 7.4), 150 mM sodium chloride, 1% Triton X-100, 1% deoxycholate, 12 mM β-glycerophosphate, 10 mM sodium fluoride, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 12 000 *g* for 15 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation with appropriate antibodies. The resulting precipitates were washed four times with buffer A and then examined by SDS–PAGE and immunoblot analysis with indicated antibodies.

Immune complex kinase assays

Cells were lysed in buffer A, and the cell lysates were subjected to immunoprecipitation with appropriate antibodies. The resulting immunoprecipitates were assayed for the activities of the indicated protein kinases, as described previously (Park *et al*, 2001; Cho *et al*, 2003). Phosphorylated proteins were separated by SDS–PAGE, and the extent of phosphorylation was quantified with a Fuji-BAS2500 phosphoimager. Bacterially expressed GST-fusion proteins of c-Jun(1-79), JNK3(K55R), and SEK1(K129R) were used as substrates for JNK/SAPK, SEK1, and SGK1, respectively.

Metabolic labeling with ^{32}P

Transfected 293T cells were transferred to phosphate-free Dulbecco's modified Eagle's medium (Invitrogen) containing [^{32}P]orthophosphate (100 μCi/ml), and were incubated for 3 h. The cells were then lysed and subjected to immunoprecipitation with anti-GST antibody. The immunoprecipitates were subjected to SDS–PAGE on a 12% gel, and phosphorylation of SEK1 was examined with a Fuji BAS2500 phosphoimager.

Clonal growth assay

MDA-MB-231 cells were plated in triplicate in 12-well culture dishes (200 cells per well) and allowed to adhere for 24 h before incubation with dexamethasone and paclitaxel, as indicated. The cells were washed twice with phosphate-buffered saline, incubated for an additional 7–10 days, fixed with 3.5% paraformaldehyde, and stained with 0.5% crystal violet in 20% methanol.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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