Antiapoptotic Effect of Serum and Glucocorticoid-Inducible Protein Kinase Is Mediated by Novel Mechanism Activating IκB Kinase

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Abstract
Serum and glucocorticoid inducible protein kinase (SGK) plays a crucial role in promoting cell survival, but the mechanisms for this response are not clear. We show that SGK is involved in the regulation of apoptosis in breast cancer cells by modulating the transcriptional activity of nuclear transcription factor-κB (NF-κB). High levels of SGK expression were observed in human breast cancer samples. When SGK was reduced the apoptotic rate increased, and increased SGK activity prevents serum withdrawal–induced apoptosis. SGK-induced cell survival was abolished by a dominant-negative form of IκB kinase β (IKKβ, K44A) or a null mutation of IKKβ in mouse embryonic fibroblast cells indicating involvement of the NF-κB pathway. Serum-induced SGK or increased expression of SGK activated NF-κB transcriptional activity, whereas small interference RNA to SGK blocked NF-κB activity. Coexpression of SGK and IKKβ significantly increased the activation of NF-κB (versus expression of IKKβ alone). Expression of dominant-negative IKKβ K44A, IκBα AA, and kinase-dead SGK (127KM) blocked the ability of SGK to stimulate NF-κB activity, suggesting that IKKβ is a target of SGK. We also show that SGK enhances the ability of IKKβ to phosphorylate endogenous IκBα in cells or recombinant glutathione S-transferase-IκBα in vitro and increases IκBα degradation; SGK physically associates with and activates IKKβ in MDA231 cells via phosphorylation of Ser181 in IKKβ. Taken together, we conclude that SGK acts as an oncogene in breast cancer cells through activation of the IκK-NF-κB pathway, thereby preventing apoptosis. Blocking SGK expression/activity represents a potential therapeutic approach for breast cancer treatment. (Cancer Res 2005; 65(2): 457-64)

Introduction
Apoptosis is an active physiologic process of gene-directed, programmed cell death. One example of apoptosis in cancer therapy is the blocking of neovascularization by antiangiogenic agents, leading to apoptosis and tumor regression (1). On the other hand, clinical studies suggest that use of antiangiogenic agents alone does not benefit many advanced breast cancers because of a mechanism that involves activation of an antiapoptotic pathway (2). Thus, understanding apoptosis and its regulation has a significant effect on cancer therapy.

Serum and glucocorticoid inducible protein kinase (SGK) has been implicated in regulation of apoptosis. For example, a newly discovered member of the SGK family, cytokine-independent survival kinase, can phosphorylate and negatively regulate proapoptotic BAD to prevent interleukin-3 withdrawal–induced cell death (3). SGK has also been shown to protect mammary epithelial cells from undergoing apoptosis following withdrawal of glucocorticoids (4). The potential for SGK in promoting growth of cancer cells is supported by reports that SGK expression is increased in primary liver cancer or ductal breast carcinoma in situ (5, 6). However, the mechanism by which SGK inhibits apoptosis in cancer is not fully explored.

Activation of NF-κB has been shown to prevent apoptosis in cancer and other cells (7, 8). The activation of NF-κB requires activation of IκK. In vivo IκK activation is preceded by phosphorylation by certain upstream kinases such as mitogen-activated protein kinase kinase kinase-1 (9–11), NF-κB–inducing kinase (12), protein kinase C (13, 14), and NF-κB–activating kinase (15). Another major signaling pathway that affects IκK complex and NF-κB activity is the PI-3 kinase pathway (16–18). For example, platelet-derived growth factor can cause transient association between Akt and IκK in vivo plus increased IKK activity with NF-κB activation and protection from apoptosis (18).

SGK belongs to the A/G protein kinase subfamily including Akt, protein kinase A, p70S6 kinase, and protein kinase C, which is activated by phosphorylation through PDK, a PI-3 kinase downstream kinase (16–18). SGK and Akt share 45% to 55% sequence identity throughout their catalytic domain (19); both kinases share a similar consensus phosphorylation site RXRXXS/T (20, 21). However, SGK has activation and expression properties that are distinct from Akt. When epithelial cells are exposed to hyperosmotic stress, heat shock, UV irradiation, oxidative stress, or steroid hormones, SGK expression and activation increase, whereas Akt remains in a nonphosphorylated/activated state (22). Activation of the glucocorticoid receptor rapidly induces SGK expression, and activated SGK, but not Akt, protects cells from apoptosis in the mammary epithelial cell line MCF 10A (4). The mechanism by which SGK protects cells from apoptosis is not clear at present. We examined if the antiapoptotic effect of SGK involves activation of NF-κB. We found that SGK physically associates with IκKβ and phosphorylates IκKβ, leading to release of NF-κB. The results identified a growth factor–responsive SGK-IKK-NF-κB survival pathway and suggest a mechanism by which SGK can suppress apoptosis of cancer cells.

Materials and Methods
Reagent. Flag-tagged protein immunoprecipitation kit, anti-Flag antibody, anti-β-actin antibody, antihemagglutinin (anti-HA) agarose–conjugated antibody, and anti-HA antibody were obtained from Sigma-Aldrich (St. Louis, MO). Antibody against IκKβ (IκKa/β H-470), IκKα, p65, β-tubulin, protein A/G agarose, and recombinant GST-IκBα were purchased from Santa

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Cruz Biotechnology (Santa Cruz, CA). Antibody against phospho-ß-ß (Ser58), phospho-IKKß (Ser177/181) was obtained from Cell Signaling Technology (Beverly, MA). Active SGK was bought from Upstate Biotechnology Inc. (Waltham, MA). Immuneaffinity purified SGK antibody was a gift from Drs. Bela Malik and D. Eaton of Emory University (Atlanta, GA). Small interfering RNA (siRNA) control and siRNA to SGK were synthesized by Dharmacon, Inc., Lafayette, CO). Insulin-like growth factor (IGF)-I was bought from Roche Applied Science (Indianapolis, IN). Vectastain Universal Quick Kit was bought from Vector Laboratory (Burlingame, CA). Luciferase assay system was purchased from Promega (Madison WI).

Cell Culture. MDA231 cells derived from human breast cancer and HEK293 cells derived from human embryonic kidney were purchased from American Type Culture Collection (Rockville, MD). MDA231/SGK cell line was created by puromycin selection after retrovirus SGK infection in MDA231 cells. Retrovirus SGK was produced by the MSCV retroviral expression system (BD Biosciences Clontech, Palo Alto, CA). Briefly, SGK cDNA was cloned in pMSCV puro vector and resultant viral DNA was infected into PT67 packaging cell line; virus-producing cell colonies were selected by puromycin. The high-titer supernatant from puromycin-transfected PT67 was used to infect MDA231 cells in the presence of 8 g/mL polybrene. Mouse embryonic fibroblast (MEF) wild-type, IKK-/- and IKK+/+ cells were kindly provided by Dr. L.M. Verma (Salk Institute, La Jolla, CA). All cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 100 unit/mL penicillin, and 100 unit/mL streptomycin (Invitrogen, Grand Island, NY). Cells were propagated at 37°C in humidified air containing 5% CO2 incubator.

Expression Vectors. pCMV4-HA-S GK was a generous gift from Dr. Hemmings, A. Brian (Friedrich Miescher-Institut, Basel, Switzerland); pRK-Flag-IKKß and dominant-negative pRK-Flag-IKKß were generously provided by Dr. J. D. Woronicz (Department of Musculoskeletal Science, Genetics Institute, Cambridge, MA); pHRNF-ß-B was generated by subcloning of four tandem repeats of HIV long terminal NF-B binding consensus site from pHR (HIV + ) -cat (23) into pGlu2 basic vector. Adenovirus NF-ß-B was from Dr. Engelhardt (University of Iowa College of Medicine, Iowa City, IA). The vectors pCMV-EGFP, pCMV-GFP, pNF-B, pMycc-luc, p53-luc, pEF-luc, and pRB-luc were bought from BD Biosciences Clontech. The expression vector pAD-CMV-track-S GK was generated by clipping SGK from pCMV4-HA-SGK and inserting it into the KpnI and EcoRV sites in the pAD-CMV-track vector, pcDNA3 was obtained from Invitrogen. The Ikkß (132-206AA) fragment was amplified from pRK-Flag-I KKß by PCR and cloned into the BamHI and HindIII sites of pGEX-KG-GST plasmid. The Ikkß mutant, GST-Ikkß (177A/181A), was generously provided by Prof. Makoto Nakashima (Nagoya City University Medical School, Nagoya, Japan).

Apoptosis Assay. Two methods were used to detect cell apoptosis. The first was done with the Annexin V-PE apoptosis detection kit 1 (BD Biosciences) according to the manufacturer’s instructions. Briefly, cells were counted, resuspended in 100 mL of binding buffer, stained with Annexin V-PE plus 7- amino-actinomycin D for 15 minutes, and analyzed by flow cytometry (Becton Dickinson, San Jose, CA). The second protocol for detecting apoptosis involved cell nuclear staining HEK293 cells or MEF cells were transfected with 0.5 g pCMV-EGFP or pCMV-Track-HA-SGK, pRK-Flag-IKKß, pRK-Flag-IKKß K44A, or siRNA, siRNA to SGK. After 24 hours, the medium was changed to serum-free medium for another 24 hours (HEK293 cells) or 48 hours (MEF cells); after gently washing with PBS, the cells were immediately fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 10 minutes before staining with 1 g/mL Hoechst 33258 (Molecular Probes, Eugene, OR) in PBS for 10 minutes as described (24). A Zeiss LSM 510 UV Meta laser-scanning confocal microscope was used at 60 magnification to count at least 500 Hoechst 33258-stained cells (blue) of transfected cells per well (GFP positive, green cells) to determine the percentage of apoptotic cells per experimental condition.

Immunoprecipitation. Cells were lysed in a lysis buffer (5 mL/L HEPES (pH 7.4), 150 mL/L NaCl, 1% Triton X-100, 10 mL/L glycerol, 1 mL/L EDTA, 1 mL/L Na3VO4, 1 mL/L phenylmethylsulfonyl fluoride, 5 mL/L aprotinin, 5 mL/L leupeptine, and 5 mL/L pepstatin). Lysates were preclarred for 30 minutes at 4°C with 20 mL/L of protein A/G agarose and 0.25 normal mouse IgG (Sigma)-HA-SGK, Flag-ß-B, or Flag-Ikkß was immunoprecipitated by incubating with agarse-conjugated anti-HA or anti-Flag antibody for 2 hours at 4°C. SGK or IKKß was immunoprecipitated by anti-SGK or anti-IKKß at 4°C overnight and then incubated with protein A/G agarose for 2 hours at 4°C. Immunocomplexes were washed five times with lysis buffer, boiled, subjected to SDS-PAGE, and transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk, the primary antibody was added and the mixture incubated and extensively washed with 0.1% Tris-buffered salt solution with Tween 20. The appropriate secondary antibody was added for 1 hour. Enhanced chemiluminescence detection was done using an ECL kit (Amersham).

b-B Kinase Assay. HEK293 cells were transiently transfected with pCMV4-HA-SGK and pRK-Flag-I KKß in 10% FBS for 24 hours. Cells were serum starved for 24 hours before adding IGF-I (10 ng/mL) for 15 minutes and then IKKß was immunoprecipitated using anti-Flag agarose-conjugated antibody. IKK activity was measured as described (25–27) by using this immunocomplex incubated with the purified recombinant GST-b-B (1-54). Briefly, the kinase activity was determined at 30°C for 30 minutes in a 30-mL reaction mixture containing 50 mL/L HEPES (pH 8.0), 10 mL/L MgCl2, 25 mL/L EGTA, 1 mL/L DTT, 10 mL/L /-glycerophosphate, 1 mL/L NaOH 1 g/mL Na3VO4, 0.1 mL/L phenylmethylsulfonyl fluoride, 10 mL/L ATP, and 10 mL/L [y-32P]ATP. Reaction products were separated by 10% SDS-PAGE, transferred to nitrocellulose, and visualized with a phosphomager.

Phosphorylation of Ikkß by SGK. The ability of SGK to phosphorylate GST-Ikkß was examined at 30°C for 30 minutes in 50 mL/L mixture plus active SGK with GST-Ikkß (132-206AA) or GST-Ikkß (177A/181A). The mixture contained 20 mL/L 4-morpholinopropanesulfonic acid (pH 7.2), 25 mL/L /-glycerol phosphate, 5 mL/L EGTA, 1 mL/L Na3VO4, 1 mL/L DTT, 2 mL/L protein kinase A inhibitor peptide and 1 mL/L microcystin-LR, 15 mL/L MgCl2, 100 mL/L cold ATP, and 10 mL/L [y-32P]ATP. Reaction products were separated by 10% SDS-PAGE, transferred to nitrocellulose, and visualized with a phosphomager.

Immunohistochemistry Staining. Second-generation (TARP-2) multimeric tumor microarray slides were obtained from the Cooperative Human Tissue Network under the Tissue Array Research Program of the National Cancer Institute (Bethesda, MD). Briefly, this multimeric tumor microarray is composed of 0.6-mm samples of 5 normal and 75 cancer of human breast tissue and some other tissues. Tissue microarray sections were dried under a low-power UV light for 2 hours. All sections were deparaffinized, dehydrated, and stained using Mouse monoclonal antibody to SGK (1:100) for 20 minutes followed by biotinylated universal second antibody for 10 minutes and then streptavidin/ peroxidase for 5 minutes before adding the peroxidase substrate for 5 seconds. Negative (normal IgG) controls were always included.

Nuclear Extract Preparation and Gel Mobility Shift Analysis of NF-ß-B Binding. Nuclear extracts of MDA231 and MDA231/SGK cells were prepared as described previously (28) and directly used for Western blotting detection of p65 nuclear translocation, b-B degradation, and NF-ß-B binding activity assay. NF-b binding activity was determined by gel mobility shift analysis. The oligonucleotide containing the NF-b binding sites was bought from Promega. The DNA-binding reaction was done at 30°C for 15 minutes in a volume of 20 mL containing 5 mg of nuclear extract, 225 mL/g bovine serum albumin, 1.0 10 counts/min of 32P-labeled probe, 0.1 mL/g poly(dex- timidinose-deoxyadenylylic acid), and 15 mL of binding buffer (12 mL/L HEPES (pH 7.9), 4 mL/L Tris, 60 mL/L KCl, 1 mL/L EDTA, 12% glycerol, 1 mL/L dithiothreitol, and 1 mL/L phenylmethylsulfonyl fluoride). After the binding reaction, the samples were subjected to electrophoresis in 1 Tris-borate EDTA buffer using 6% native polyacrylamide gels.

Statistics. Values were considered statistically significant at P < 0.05.
Results

**SGK Level Is Increased in Breast Cancer Tissue.** SGK protein level in the TARP-2 breast cancer tissue array obtained from National Cancer Institute was examined by immunohistochemistry using anti-SGK. A high level of SGK was found in 29 of 38 breast cancer tissue samples. SGK was low or nondetectable in normal breast tissue samples (5 of 5; Fig. 1). These data suggest that up-regulated SGK may play a role in controlling breast cancer development.

**SGK Protects Cancer Cells from Apoptosis following Serum Withdrawal.** To address the biological relevance of high SGK expression in cancer cells, we examined whether SGK expression inhibits apoptosis in the breast cancer cell line MDA231. After withdrawing serum, we found a low level of SGK in MDA231 cells. Cells treated with serum (0-7 hours) exhibited an increased level of SGK (Fig. 2A). Apoptosis occurring in these conditions was measured by FACS-based Annexin V staining. As shown in Fig. 2B and C, serum withdrawal had a 16.5% apoptotic rate in contrast to 5.34% in the presence of serum in MDA231 cells. To examine if a high SGK level alone protects cells from apoptosis, we expressed pCMV-track-SGK in HEK293 cells (cells expressing SGK are green) or GFP alone. After serum withdrawal, SGK expression dramatically reduced apoptosis (by 90%) compared with control cells (data not shown). To provide a direct link of SGK to apoptosis, we suppressed 68.6% SGK by siRNA SGK (Fig. 2E) in MDA231 cells; the apoptotic rate was 64.24% higher than in control cells (transfected with siRNA control) in the absence of serum (Fig. 2D). Thus, SGK exhibits antiapoptotic effects.

**IKK3 and SGK Interact to Inhibit Apoptosis.** To evaluate if SGK suppresses apoptosis via IKK, we expressed the wild-type pCMV-track-SGK in HEK293 cells with or without the dominant-negative IKKβ (pRK-Flag-IKKβ K44A). After serum-free conditions for 24 hours, apoptosis was measured by the change in nuclei morphology using confocal microscopy. Expression of wild-type SGK was associated with a significant reduction in the number of apoptotic cells, 40 of 1,110 SGK-transfected green cells undergo apoptosis (Fig. 3A and B, lane 2). In contrast, expression of SGK and a mutant IKKβ (IKKβ K44A) blocked the antiapoptotic ability of SGK (290 of 564 cells undergo apoptosis (Fig. 3A and B, lane 4)). Thus, the antiapoptotic effect of SGK involves IKK, suggesting a potential role for the antiapoptotic NF-κB pathway.

To address the specificity of the IKK that is involved in the SGK antiapoptotic pathway, MEF wild-type, IKKα−/− or IKKβ−/−/− cells (Fig 3C) were transfected with pCMV-track-HA-SGK or control pCMV-track. Subsequently, the cells were subjected to serum-free media for 48 hours and then fixed and stained with Hoechst 33258. The apoptotic cells were measured by the change in nuclear morphology. Expression of wild-type SGK was associated with a significant reduction in the number of apoptotic cells in MEF cells. However, expression of SGK in either MEF cells that are IKK or IKKβ null (Fig. 3E) does not provide protection from apoptosis when serum is withdrawn (Fig. 3C and D). Thus, the antiapoptotic effect of SGK involves IKK, suggesting a potential role for antiapoptotic NF-κB pathway.

**SGK Increases NF-κB Activity through an IkB Kinase.** Next, we evaluated the effect of SGK expression on NF-κB activation using a luciferase-promoter assay. When HEK293 cells were cotransfected with wild-type SGK and pNF-κB-luc or with four other report vectors (pMyc-luc, p53-luc, pE2F-luc, and pRb-luc), we found that SGK expression dramatically increased the NF-κB promoter activity. In sharp contrast, SGK had little or no influence on the luciferase activity driven by promoters for Myc, p53, E2F, or Rb (Fig. 4A). The selective enhancement of the NF-κB–responsive promoter suggests that SGK specifically induces NF-κB activity. As a control, we found that SGK does not increase mutant NF-κB activity (Fig. 4B). In this experiment, MDA231 cells were transfected with SGK and wild-type or mutant HIV-NFκB-luc, a construct consisting of four HIV-NFκB binding sites (23), then treated with 10% serum for 24 hours. FBS induced a 5-fold increase in HIV-NFκB-luc promoter activity in the presence of SGK (Fig. 4B) but failed to increase mutant HIV-NFκB activity with SGK expression (Fig. 4B).

To address whether SGK increases NF-κB under physiologic conditions, we infected MDA231 cells with adenovirus NF-κB-luc. After serum starvation, the cells were treated with 10% FBS to increase the SGK level. The luciferase assay indicated that induction of SGK with serum resulted in a higher level of NF-κB activity (Fig. 4C). To examine the specificity of SGK-increased NF-κB, we blocked endogenous SGK by transfection of siRNA of SGK into MDA231 cells and used a nonspecific siRNA as control. The results indicate that expression of SGK increased NF-κB activity 82% over the expression of siRNA control (control), whereas transfection of siRNA against SGK blocked 90% of NF-κB activity compared with siRNA control (Fig. 4D). Moreover, serum failed to activate NF-κB when SGK is blocked by the siRNA SGK (Fig. 4E).

**Figure 1.** SGK expression in primary breast cancer tissues. Immunostaining of a breast cancer tissue array with anti-SGK or control IgG. A and B, representative normal breast tissue stained with anti-SGK. C and D, representative of breast cancer tissue with high SGK staining. E and F, negative staining of breast cancer tumor tissue with IgG.
NF-κB is normally sequestered in the cytoplasm by its interaction with the inhibitory protein, IκB (29). Phosphorylation of IκB by IKK leads to IκB degradation with release of NF-κB, which translocates to the nucleus to initiate gene transcription. To test if the activation of NF-κB transcriptional activity by SGK involves IKK, we found that HEK293 cells expressing SGK or IKKβ alone yielded a 6- to 8-fold increase in NF-κB activity, respectively (Fig. 4F). However, the combination of SGK and IKKβ expression produced an additive effect (i.e., a 12-fold increase over control values; Fig. 4F). In contrast, kinase-dead SGK or dominant-negative IKKβ K44A inhibited SGK-induced NF-κB activity (Fig. 4F). The nondegradable serine-to-alanine mutant IκB (IκB-AA) also inhibited the activation of NF-κB (Fig. 4G). These results suggest IKK is acting downstream of SGK to mediate NF-κB activation.

**SGK Increases IκBα Degradation, p65 Nuclear Translocation, and NF-κB DNA-Binding Activity.** To provide additional evidence for SGK increased NF-κB transcriptional activity, IκBα degradation, p65 nuclear translocation, and NF-κB binding were analyzed. We compared MDA231 cells with the MDA231/SGK cell line treated with or without serum. Serum treatment for 30 minutes increased NF-κB binding activity in MDA231/SGK cells compared with MDA231 cells (Fig. 4H). Note that in serum-free condition, there is also a higher NF-κB binding activity in SGK cells. Consistent with the gel shift results, we found higher p65 nuclear translocation and IκBα degradation in MDA231/SGK cells than MDA231 cell (Fig. 4I and J).

**SGK Enhances IKK Activity in Cultured Cells.** To examine if SGK regulates IKK activity and phosphorylation of IκBα at Ser32 and Ser36 (30), we overexpressed wild-type IκBα tagged with Flag in HEK293 cells that were transfected with or without the wild-type SGK and with or without wild-type Flag-IKKβ. IκBα was immunoprecipitated by anti-Flag and detected by Western blotting with a phospho-IκBα antibody (phospho-Ser32). As shown in Fig. 5A, IKK expression alone induced minimal phosphorylation of IκBα at Ser32 (Fig. 5A, lane 3), whereas modest expression of IKKβ alone induced a limited degree of Ser32 phosphorylation (Fig. 5A, lane 2). In contrast, expression of SGK and IKKβ together dramatically increased IκBα phosphorylation (Fig. 5A, lane 4) indicating that a high SGK can increase IKK activity in HEK293 cells. There also was phosphorylation of IκBα in response to SGK occurring in a SGK dose-dependent manner (Fig. 5B). Next, we activated SGK by 10 ng/ml IGF-I and, as shown in Fig. 5C, IκBα phosphorylation was significantly increased when SGK was activated.

To confirm that SGK activates IKK in vivo, we did an in vitro IKK kinase assay by measuring phosphorylation of purified recombinant protein GST-IκBα (1-54) in the presence of [γ-32P]ATP. First, IKK was immunopurified from HEK293 cells that has been transfected with SGK and with or without wild-type Flag-IKKβ. As shown in Fig. 5D, SGK increases IKK-mediated phosphorylation of IκBα, unless IKK was mutated. Second, we immunoprecipitated HA-SGK and Flag-IKKβ from individual HEK293 cells transfected with HA-SGK or Flag-IKKβ, respectively. As shown in Fig. 5E, combining the two immunocomplexes increased the phosphorylation of GST-IκBα (1-54; Fig. 5E). These results indicate that SGK could act upstream to activate IKK.

**SGK Associates with and Phosphorylates IKK.** To test if SGK could increase IKK activity by forming a protein-protein interaction complex, we transfected HEK293 cells with HA-SGK and
Flag-IKKβ. In immunocomplexes obtained using an anti-HA antibody, we found the presence of IKKβ (Fig. 5F, lane 2). Likewise, the association of SGK and IKK was also detected in HA-SGK-transfected MDA231 cells (Fig. 5F, lane 1). To correlate the association of SGK with IKKβ in untransfected MDA231 cells, we treated serum-starved cells with 10% FBS to increase SGK expression. The cell lysate was immunoprecipitated with anti-SGK or anti-IKKβ antibody. SGK has a molecular weight close to that of the IgG heavy chain, so we labeled the SGK antibody with an antibody labeling kit (IRDye 800CW, LI-COR Biosciences, Lincoln, NE) and did not use a secondary antibody. As shown in Fig. 5G, IKKβ was present in SGK complexes and SGK was present in IKKβ-based complexes. These observations show that SGK and IKKβ physically associate in MDA231 cancer cells.

To determine if SGK can phosphorylate the specific activation site in IKKβ, we purified GST-IKKβ wild-type (132 to 206AA) and serine mutant peptide (S177A/S181A) using a glutathione sepharose 4B column. Phosphorylation of these IKKβ peptide fusion proteins by recombinant active SGK was measured in the presence of [γ-32P]ATP. As shown in Fig. 5H, SGK phosphorylated the wild-type recombinant IKKβ protein but not the mutant IKKβ (S177A/S181A; Fig. 5H). Finally, we expressed IKKβ with or without SGK in HEK293 cells and found that expression of SGK stimulated phosphorylation of Ser177/181 of IKKβ, which was measured by phospho-IKKβ 177/181 antibody (Fig. 5I). These results show that SGK can phosphorylate IKK both in vitro and in cultured cells.

Discussion

Our results show for the first time that SGK can directly activate IKK and stimulate NF-κB activity. A high level of expression of SGK in breast cancer cells suggests that this kinase may function to protect tumor cells from apoptosis, hence, act as an oncogene. These findings provide a potential explanation of why strategies such as antiangiogenic therapy are only partially successful in treating breast cancer. The high level of SGK in breast cancer with antiapoptotic properties would counteract the apoptotic response that should occur when blood supply is reduced. In short, an elevated SGK in advanced tumor could protect cancer cells from serum withdrawal–induced apoptosis.

Several studies show that SGK is involved in cell survival. For example, gene expression analysis shows that SGK expression is increased in tumors that include liver and breast cancer (5, 6). There is also evidence that SGK protects cells during growth factor withdrawal (3, 4, 31). We found that a high level of SGK expression is present in many human breast cancer tissues, whereas downregulation of SGK by siRNA SGK will enhance serum withdrawal–induced apoptosis of the breast cancer MDA231 cell while SGK expression inhibits apoptosis.

How does an actual SGK prevent apoptosis? Activated NF-κB leads to transcriptional activation of genes whose products block...
apoptosis, including members of the Bcl-2 family, cellular inhibitors of apoptosis, and others (32). Previous studies have identified targets for this response in phosphorylation and negative regulation of the proapoptotic BAD and Forkhead transcriptional factors (3, 31). We provide evidence that IKK-NF-κB is involved and that a high level of SGK can increase NF-κB transcription activity by 82%, whereas knockdown of SGK in MDA231 cell by siRNA SGK will block NF-κB transcriptional activity by 92%.

How does SGK stimulate NF-κB transcriptional activity? We showed for the first time that SGK physically associates with IKKβ in cultured cells and, more important, phosphorylates IKKβ in vitro to enhance IKK activity, thus increasing IκB phosphorylation to cause its degradation and increasing p65 translocation and NF-κB DNA binding. Our results indicate that SGK directly activates IKKβ to stimulate NF-κB. However, we found that the SGK survival effect is abolished in IKKα MEF cells (Fig. 3C and D), suggesting this response also requires the presence of IKKα. This is not surprising because others have shown that IKKα and IKKβ are both necessary for the expression of NF-κB–dependent, induced genes in cells (33). Moreover, several studies indicated that IKKα is also essential to contribute NF-κB activation by modification of chromatin, that is, phosphorylation of histone H3 to modulate chromatin accessibility at NF-κB–responsive promoters (34–36). In line with these considerations, we and others find that knockout of IKKα leads to a 70% reduction in basal NF-κB activity compared with wild-type cells (data not shown). We conclude that both IKKα and IKKβ are required for maximal activation of NF-κB, but our finding that there is a physical association of SGK with IKKβ and phosphorylation of IKKβ point to a new mechanism by which SGK promotes cell survival.

It has been reported that PDGF-induced Akt activation causes a transient association of Akt with IKK to stimulate the NF-κB signaling pathway (17, 18). Because the kinase domain of SGK exhibits high (80%) homology with Akt and both are activated by

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**Figure 4.** SGK increases NF-κB activity. A, HEK293 cells were cotransfected with SGK and the indicated promoter-reporter luciferase construct and luciferase activity was detected. Columns, mean from four individual experiments done in triplicate; bars, SE. B, MDA231 cells were transfected with wild-type or mutant HIV-NF-κB–luc and SGK or cDNA3; after serum starvation for 24 hours, cells were treated with 10% serum for another 24 hours before luciferase activity was determined. C, MDA231 cells infected with adenovirus NF-κB–luc and treated with 10% serum for different times after starvation. Luciferase activity was measured by a luminometer. D, MDA231 cells were cotransfected with NF-κB–luc with SGK, siRNA SGK, or siRNA control, and luciferase activity was determined. Columns, percentage changes of SGK (lane 1) or siRNA SGK compared with control (siRNA control), % change = ([(SGK or siRNA to SGK-siRNA control)/siRNA control] × 100). E, siRNA SGK blocks FBS-stimulated NF-κB activity. MDA231 cells were cotransfected with siRNA control or siRNA SGK with NF-κB–luc. After 24 hours serum starvation, cells were treated with or without 10% serum for 24 hours and luciferase activity was measured. F, HEK293 cells were cotransfected as indicated plus NF-κB–luc constructs; luciferase activities were determined. G, SGK–mediated NF-κB activity was blocked by the dominant-negative IκBα. H, SGK increases NF-κB DNA binding. The nuclear protein was isolated from MDA231 and MDA231/SGK cells under serum-free or 10% serum (30 minutes) conditions. These proteins were used for NF-κB DNA-binding assay. The competition of cold probe will block the specific binding. I, nuclear proteins from H were used for Western blotting with p65 to determine if there was p65 nuclear translocation in the cells with or without SGK. β-Tubulin was used as a loading control. J, total lysates of MDA231 and MDA231/SGK cells were used for Western blotting to detect IκBα degradation. β-Actin used as a loading control.
phosphorylation via PDK1 and PDK2 kinases, which are downstream from PI-3 kinase (21), our data suggest an important alternative pathway for PI-3 kinase–mediated NF-κB activation. This is an important finding because there are differences between SGK and Akt, including recognition sites for phosphorylation. For example, SGK phosphorylates Ser\(^{315}\) of the FKHR1 transcription factor, whereas Akt favors Ser\(^{325}\) (31). Moreover, stimulation of SGK expression and activity is different from Akt because epithelial cells exposed to multiple types of environmental stress activated SGK, whereas Akt remained in a nonphosphorylated state (22). In short, SGK could act as an alternative signaling pathway for PI-3 kinase–mediated NF-κB transcriptional factor activation.

In summary, our results point to novel mechanisms that link SGK to cell survival. SGK directly activates IKK to stimulate NF-κB activity and the high level of expression of SGK in breast cancer cells suggests that this kinase is functioning to promote tumor cell survival and hence, acts as an oncogene.

**Acknowledgments**


**Grant support:** NIH grant ROI HL 70762, American Heart Association Scientist Development grant (J. Du), and University of Texas Medical Branch John Sealy endowed grant.

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