Induction of Apoptosis by the Ste20-like Kinase SLK, a Germinal Center Kinase That Activates Apoptosis Signal-regulating Kinase and p38*  

Received for publication, October 31, 2005, and in revised form, November 28, 2005. Published, JBC Papers in Press, November 29, 2005, DOI 10.1074/jbc.M511744200  

Wen Hao, Tomoko Takano1, Julie Guillemette, Joan Papillon, Guohui Ren, and Andrey V. Cybulsky1,2  

From the Department of Medicine, McGill University Health Centre, Montreal, Quebec H3A 1A1, Canada  

Expression and activity of the germinal center kinase, Ste20-like kinase (SLK), are increased during kidney development and recovery from ischemic acute renal failure. In this study, we characterize the activation and functional role of SLK. SLK underwent dimerization via the C-terminal domain, and dimerization enhanced SLK activity. In contrast, the C-terminal domain of SLK did not dimerize with a related kinase, Mst1, and did not affect Mst1 activity. Phosphorylation/dephosphorylation of SLK was not associated with changes in kinase activity. SLK induced phosphorylation of apoptosis signal-regulating kinase-1 (ASK1) and increased ASK1 activity, indicating that ASK1 is a substrate of SLK. Moreover, SLK stimulated phosphorylation of p38 mitogen-activated protein kinase via ASK1, but not c-Jun N-terminal kinase nor extracellular signal-regulated kinase. Chemical anoxia and recovery during re-exposure to glucose (ischemia–reperfusion injury in cell culture) stimulated SLK activity. Overexpression of SLK enhanced anoxia/recovery-induced apoptosis, release of cytochrome c, and activities of caspase-8 and -9, and apoptosis was reduced significantly with p38 and caspase-9 inhibitors. Induction of the endoplasmic reticulum stress response by anoxia/recovery or tunicamycin (monitored by induction of Bip or Grp94 expression, phosphorylation of eukaryotic translation initiation factor 2α subunit, expression of CHOP, and activation of caspase-12) was attenuated in cells that overexpress SLK. Thus, SLK is an anoxia/recovery-dependent kinase that is activated via homodimerization and that signals via ASK1 and p38 to promote apoptosis. Attenuation of the protective aspects of the endoplasmic reticulum stress response by SLK may contribute to its proapoptotic effect.

The mammalian germinal center kinases (GCKs)3 comprise a family of protein kinases that are homologous to Ste20 of S. cerevisiae (1–4). All GCKs possess N-terminal kinase domains and C-terminal regulatory domains, some of which are extensive. Originally, the GCKs were divided into two groups. The group I GCKs interact with mitogen-activated protein kinase (MAPK) kinase-1, and they activate the c-Jun N-terminal kinase (JNK) pathway but not extracellular signal-regulated (ERK) or p38 pathways. Several group I GCKs are activated by tumor necrosis factor, and their biology has been reviewed previously (2). The original group II GCKs have been more recently subdivided into seven families (i.e. groups II–VIII) (3). These GCKs are expressed ubiquitously, with the exception of lymphocyte-oriented kinase (5), which is expressed in lymphocytes. Some of these GCKs can be activated in vivo by various stresses (e.g. heat-shock, arsenite, staurosporine, ischemic injury, or ATP depletion) (6, 7). Most of the group II–VIII GCKs do not fit into the well defined MAPK pathways. These kinases do not activate ERK, JNK, or p38 pathways, although there may be one or two exceptions (8–10).

Kidney development is dependent on growth factors, activation of growth factor receptors, and interaction of cells with extracellular matrices (11). During development, there is proliferation of renal cells as well as apoptosis (12, 13), and tight control of cell growth and apoptosis is essential for formation of normal renal anatomy and cell differentiation. In the mature kidney, interruption of blood flow leads to ischemia, which is a major cause of acute renal failure (14, 15). Ischemia typically injures renal tubular epithelial cells and also glomerular cells and is characterized by ATP depletion and other metabolic derangements. Reperfusion (upon restoration of blood flow) is associated with production of reactive oxygen species. Ischemia–reperfusion may lead to apoptosis or necrosis of kidney cells, but the cells that survive the insult may undergo a process resembling development, which includes dedifferentiation, re-entry into the cell cycle, and proliferation to replace the dead cells. In cultured cells, chemical anoxia and glucose re-exposure recapitulates the ATP depletion and production of reactive oxygen species seen during ischemia in vivo.

During screening of rat fetal kidney mRNA for protein-tyrosine kinases, we discovered a mRNA consistent with the group V GCK, Ste20-like kinase (SLK, or SK2 in the rat). Expression of SLK mRNA and protein as well as kinase activity were increased in rat fetal kidney homogenates (embryonic days 17–21), as compared with adult control kidneys (16). Moreover, in adult kidneys subjected to ischemia–reperfusion injury, SLK mRNA and protein expression and kinase activity were increased, as compared with untreated contralateral control kidneys. By immunohistochemistry, SLK expression was evident mainly in the cytoplasm of tubular epithelial cells in fetal and adult kidneys, and there was some expression in developing and mature glomerular epithelial cells (GECs, or podocytes). Thus, SLK is a renal epithelial protein kinase, whose expression and activity are increased during development as well as recovery from acute renal failure, where injured tubular epithelial cells may regenerate by recapitulating developmental processes. Localization in GECs also suggests a possible role in glomerulogenesis or glomerular injury. In cell culture, incubation of epithelial cells with serum or exposure to chemical anoxia, followed by re-exposure to glucose (in vitro ischemia-reperfusion), increased SLK activity. Overex-

---

1 To whom correspondence should be addressed: Division of Nephrology, Royal Victoria Hospital, 687 Pine Ave. W., Montreal, Quebec H3A 1A1, Canada. Tel.: 514-398-8148; Fax: 514-843-2815; E-mail: andrey.cybulsky@mcgill.ca.

2 *This work was supported by Research Grants from the Canadian Institutes of Health Research and the Kidney Foundation of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3 Recipient of a scholarship from the Fonds de la Recherche en Santé du Québec.

4 To whom correspondence should be addressed: Division of Nephrology, Royal Victoria Hospital, 687 Pine Ave. W., Montreal, Quebec H3A 1A1, Canada. Tel: 514-398-8148; Fax: 514-843-2815; E-mail: andrey.cybulsky@mcgill.ca.

5 The abbreviations used are: GCK, germinal center kinase; ASK1, apoptosis signal-regulating kinase-1; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GEC, glomerular epithelial cell; GST, glutathione S-transferase; SLK, Ste20-like kinase; GST-SLK(CT), GST-SLK C-terminal domain fusion protein; HA, hemagglutinin antigen epitope tag; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MDCK, Madin-Darby canine kidney.

---

*This work was supported by Research Grants from the Canadian Institutes of Health Research and the Kidney Foundation of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
pression of SLK in epithelial cells attenuated increases in cell number under normal culture conditions, probably at least in part due to apoptosis, and, in the setting of anoxia, exacerbated cell death. Thus, activation of SLK may attenuate cell proliferation during kidney development or recovery from ischemic injury. Furthermore, activation of SLK may actually exacerbate injury or delay recovery. In other cell lines, transient overexpression of SLK induced apoptosis (9, 10), and in these studies the authors were unable to generate stably transfected cell lines, since SLK overexpression appeared to be cytotoxic. Epidermal growth factor, anisomycin, and hyperosmolality did not stimulate SLK activity in transfected COS-7 cells, and transfection of constitutively active Ras, Rac, or cell division cycle-42 also did not enhance SLK activity (17). These characteristics are generally in keeping with other group II–VIII GCKs (2).

The regulation and signaling by group II–VIII GCKs is poorly understood (2, 3). By analogy to some non-GCKs (18, 19), our results suggest that SLK may be, in part, regulated via changes in mRNA/protein expression (e.g. transcriptional or post-transcriptional regulation) (20–22). Many protein kinases, including some GCKs, are believed to be regulated by phosphorylation. For example, in vitro, Mst1 can be activated by phosphorytase 2A, whereas activities of SOK1 and SLK are reported to be dependent upon autophosphorylation (6, 9). GCKs may possess significant basal activity when immunoprecipitated from endogenous sources or when overexpressed (2, 17). SLK (and other GCKs) have a coiled-coil structure in their C-terminal domains, and certain proteins with the coiled-coil structure are known to oligomerize by means of this structure (23). Thus, SOK1 and Mst1 spontaneously homodimerize in vivo. Since SLK shows some constitutive activity when overexpressed in COS-7 cells (17), it is reasonable to propose that increased expression of SLK in the developing kidney or after ischemic injury may facilitate interaction of SLK with itself or other coiled-coil proteins and enhance kinase activity. The present study demonstrates that SLK is an ischemia-reperfusion-dependent protein kinase that may be activated via homodimerization and that signals via apoptosis signal-regulating kinase-1 (ASK1) and p38 MAPK to promote apoptosis. Furthermore, SLK attenuates the endoplasmic reticulum (ER) stress response, which may contribute to its proapoptotic effect.

**EXPERIMENTAL PROCEDURES**

**Materials and Plasmid Construction**—Tissue culture and molecular biology reagents were obtained from Invitrogen. Electrophoresis and immunoblotting reagents were from Bio-Rad. Myelin basic protein, calyculin A, caspase-8 substrate (granzyme B substrate I), propidium iodide, caspase-9 substrate II, and LEHD-CHO were obtained from Calbiochem. Bisbenzimide H33342 fluorochrome, [32P]ATP (3 Ci/mmol) and [3H]thymidine (2 Ci/mmol) were purchased from PerkinElmer Life Sciences. Bisbenzimide H33342 fluorochrome, propidium iodide, caspase-8 substrate (granzyme B substrate I), caspase-9 substrate II, and LEHD-CHO were obtained from Calbiochem. Sabourin (University of Ottawa).

To construct Myc-His-tagged SLK, the cDNA of full-length SLK plus the 3’-untranslated region (kindly provided by Dr. T. Nagase, Kazusa DNA Research Institute, Japan) was subcloned into the expression vector pcDNA3.1/Myc-His(−). (This construct will express full-length SLK but not the Myc-His tag.) The portion of hSLK between a unique 3426-bp Xhol site and the 3’ NotI site in the vector was excised to remove the stop codon at 3970 bp (plus the 3’-untranslated region) and was replaced with a 484-bp PCR fragment (primers 5’-AGAGCTCGGAAGCTGCAA and 3’-TGTTGAGCATGCGCCCGAAAAC-TGA) that excludes the stop codon and allows transcription of SLK tagged with Myc-His at the C terminus. Expression of Myc-His-SLK in transiently transfected COS-1 cells is presented in Fig. 1A.

**Cell Culture and Transfection**—COS-1 cells were employed for studies involving transient transfection. For stable transfection of SLK, we employed Madin-Darby canine kidney (MDCK) cells (a kidney tubule epithelial cell line) and rat GECs. Some studies were carried out in both MDCK cells and GECs (16); however, some antibodies did not cross-react with canine proteins, and these experiments were thus carried out in MDCK cells and COS cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. GEC culture and characterization has been published previously (24–26). GECs were cultured in 1% medium. Studies were done with cells between passages 8 and 60. Stable transfection of GECs with HA-SLK cDNA was performed by the calcium phosphate technique, as described previously for cytotoxic phospholipase A2 and other proteins (25, 26). Stable transfection of MDCK cells with HA-SLK cDNA was performed using Fugene-6, according to the manufacturer’s instructions. After primary selection with G418, stably transfected clones were selected by immunoblotting with anti-HA antibody.

Stable transfection of MDCK cells with HA-SLK cDNA is shown in Fig. 1B. Immunoblotting with anti-HA antibody demonstrated that some but not all transfected clones stably overexpress HA-SLK (Fig. 1B). The amounts of overexpression were variable, and even the highest levels of overexpression were relatively low. Clone 11, which shows the highest level of overexpression (Fig. 1, B and C), was selected for further studies. Clones of GECs that stably express HA-SLK (Fig. 1C) were characterized previously (16).

**Immunoblotting, Immunoprecipitation, and Immune Complex Kinase Assays**—Methods for immunoblotting and immunoprecipitation were described previously (16, 25, 26). In some experiments, protein loading was verified by Ponceau staining of nitrocellulose membranes. The membranes were incubated with 0.1% Ponceau-S in 1% acetic acid for 5 min at 22 °C. Membranes were then destained with 1% acetic acid for 5 min at 22 °C. The membranes were incubated with 0.1% Ponceau-S in 1% acetic acid for 5 min at 22 °C. Membranes were then destained with 1% acetic acid. For immune complex kinase assays (16), cell proteins (~0.5 mg) were solubilized in buffer containing 0.5% Triton X-100, 50 mM β-glycerophosphate, 10 mM MgCl2, 2 mM dithiothreitol, 20 μM leupeptin, 20 μM pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM Na2VO4, 3 mM EDTA, 3 mM EGTA, pH 7.5 (4°C). Proteins were immunoprecipitated with primary antibody IgG (2 h, 4°C) or nonimmune IgG in controls (background), followed by absorption with agarose-coupled protein A (1 h, 4°C). The immunoprecipitates were then mixed with 20 μl Hpes, pH 7.2, 20 mM β-glycerophosphate, 10 mM MgCl2, 1 mM dithiothreitol, 0.5 mM Na2VO4, 0.5 mg/ml bovine brain myelin.
basic protein, and 20 μM [γ-32P]ATP (5 μCi). GST-SLK(CT) (0.2 μg) was added in some experiments. After a 5-min incubation at 30 °C, the reaction was terminated by the addition of Laemmli buffer, and the mixture was subjected to SDS-PAGE and autoradiography. Densitometry of immunoblots or autoradiograms was performed using NIH Image software. For immunoblotting, equal amounts of proteins were loaded into each lane of a gel. Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

**Measurement of Apoptosis, Cell Number, [3H]Thymidine Incorporation, and Cell Cycle Analysis—** Apoptosis was quantified by Hoechst H33342 and propidium iodide staining, as described previously (16, 27). Nuclei of apoptotic cells were stained brightly with H33342 dye and stained negatively with propidium iodide. Analysis was performed using the University of Texas Science Center at San Antonio Image Tool program. In some experiments, apoptosis was confirmed with a DNA laddering assay, as described previously (27). Intracellular caspase-8 and -9 activities were measured using a colorimetric assay with p-nitroaniline-labeled substrates, as described previously (27). Changes in cell number were determined by trypsinization of adherent cells, followed by visual counting of cells in a hemacytometer (27).

To measure [3H]thymidine incorporation, cells were plated into 24-well plates at 5,000 cells/well. At 24 or 48 h, 0.5 μCi of [3H]thymidine was added to wells for 6 h. Cells were washed four times with phosphate-buffered saline and were solubilized with 0.1 M NaOH. Lysates were added to scintillation fluid, and radioactivity was quantified in a β-scintillation counter.

For cell cycle analysis, cells were maintained at confluence for 5–7 days and were incubated in serum-poor medium (0.5% fetal calf serum) for the final 48 h. Cells were then replated in serum-replete medium and were trypsinized at 24 h. The cells were resuspended in 1 ml of phosphate-buffered saline and were added to 4.0 ml of 100% ethanol (−20 °C). Cells were centrifuged and resuspended in 0.125 ml of 1.12% sodium citrate, containing 2.5 μg of RNase A, and were incubated for 30 min at 37 °C. Then 0.375 ml of 1.12% sodium citrate containing 0.05 mg/ml propidium iodide was added for 30 min at 22 °C. DNA content was measured by fluorescence-activated cell sorting and was analyzed using the CellQuest program (BD Biosciences). Data are presented as the percentage of cells in G1 phase (2N DNA content), G2/M phase (4N DNA content), and apoptosis (<2N DNA content).

**Statistics—** Data are presented as mean ± S.E. The t statistic was used to determine significant differences between two groups. One-way analysis of variance was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the t statistic and adjusting the critical value according to the Bonferroni method.

**RESULTS**

**Dimerization of the SLK C-terminal Domain Increases SLK Activity—** The first series of experiments addressed the activation of SLK. Analysis of SLK using the SMART program (28) shows an α-helical coiled-coil structure (23) in the C-terminal domain. Certain proteins with coiled-coils are believed to dimerize/oligomerize via this structure. To address the potential role of homodimerization, homogenates of MDCK cells stably transfected with HA-SLK were incubated with or without purified GST-SLK(CT). The next series of experiments addressed the role of phosphorylation in regulating SLK activity. Phosphorylation of SLK was induced by incubation of MDCK cells that overexpress HA-SLK with calyculin A, a serine/threonine protein phosphatase inhibitor (which inhibits phosphatases 1 and 2). Calyculin A was proapoptotic in MDCK cells, and the amounts of protein loaded onto gels.
Role of SLK in Renal Ischemia-reperfusion Injury

FIGURE 2. Activation of SLK. A, dimerization of SLK C-terminal domain. Samples include homogenates of MDCK cells stably transfected with HA-SLK mixed with (lanes 2 and 3) or without purified GST-SLK(CT) (lanes 1, 4, and 5). Proteins were immunoprecipitated with anti-HA antibody (+) or absorbed with glutathione (glut)-agarose (−), and were then immunoblotted with anti-SLK antibody. Anti-HA immunoprecipitation (IP) specifically recovered GST-SLK(CT). B, dimerization of SLK C-terminal region increases SLK activity. Homogenates of MDCK cells stably transfected with HA-SLK were incubated with or without purified GST-SLK(CT). Proteins were immunoprecipitated with mouse anti-HA antibody or normal mouse IgG (controls), and kinase activity was monitored by phosphorylation of myelin basic protein (MBP) in the presence of (γ-32P)ATP (5 min, 30°C). Top, representative autoradiogram; bottom, densitometric quantification (the bars in the graph are in the same order as the first and second lanes in the autoradiogram; MBP values were determined by subtracting mIgG controls from anti-HA). Myelin basic protein phosphorylation was increased in the presence of GST-SLK(CT) (t /p < 0.01 versus HA-SLK, n = 6). C, calcinulin A (CA) increases SLK phosphorylation, as compared with untreated (Untr) cells, and calf intestinal alkaline phosphatase (Pase) treatment reverses phosphorylation (t /p < 0.001 versus calcinulin A + calf intestinal alkaline phosphatase, p < 0.045 versus untreated; n = 3). MDCK cells stably transfected with HA-SLK were incubated with or without 50 nM calcinulin A for 30 min. Lysates were immunoprecipitated with anti-HA antibody (+) or normal mouse IgG (controls −), and the immunoprecipitates were incubated with (γ-32P)ATP. Immune complexes were incubated with or without phosphatase (25 min) for 30 min at 37°C. SLK phosphorylation was analyzed by autoradiography. D, effect of calcinulin A on SLK and phosphatase treatment on SLK activity. MDCK cells stably transfected with HA-SLK were incubated with or without 50 nM calcinulin A for 30 min. Lysates were immunoprecipitated with mouse anti-HA antibody (normal mouse IgG in controls). Immunoprecipitates were incubated with or without phosphatase (25 min) for 30 min at 37°C. Kinase activity was monitored by phosphorylation of myelin basic protein in the presence of (γ-32P)ATP. Although calcinulin A and calcinulin A + phosphatase tended to increase SLK activity, the differences among groups were not statistically significant (n = 4).

FIGURE 3. Effect of GST-SLK(CT) on Mst1 activity. A and B, GST-SLK(CT) does not heterodimerize with Mst1. Samples include homogenates of COS cells transiently transfected with FLAG-Mst1 cDNA and purified GST-SLK(CT), alone or in combination. Proteins were immunoprecipitated with anti-FLAG antibody (4) or absorbed with glutathione (glut)-agarose (8) and were then immunoblotted with anti-SLK antibody. Anti-HA immunoprecipitation (IP) specifically recovered GST-SLK(CT). B, dimerization of SLK C-terminal region increases SLK activity. Homogenates of COS cells transiently transfected with FLAG-Mst1 were incubated with or without purified GST-SLK(CT). Proteins were immunoprecipitated with mouse anti-FLAG antibody or glutathione antibody (controls), and kinase activity was monitored by phosphorylation of myelin basic protein in the presence of (γ-32P)ATP (5 min, 30°C). Top, autoradiogram; bottom, densitometry of myelin basic protein phosphorylation. Mst1 and Mst1 + GST-SLK(CT), n = 10.

apotoposis reached 94–100% (4 h of incubation). Calcinulin A (50 nM) increased SLK phosphorylation, as compared with untreated cells (Fig. 2C). Subsequent treatment with calf intestinal alkaline phosphatase resulted in dephosphorylation of SLK (Fig. 2C). These changes in SLK phosphorylation/dephosphorylation did not, however, correlate with changes in SLK activity (Fig. 2D), indicating that SLK activity is not dependent on phosphorylation, at least under these experimental conditions.

ASK1 Is a Substrate of SLK—The downstream targets of most group II–VIII GCKs remain enigmatic. We analyzed the cDNA sequence of the SLK catalytic domain using a computer program that predicts potential protein kinase substrates (29). The program predicted an optimal target amino acid sequence of V(R/K)XS(F/L)V(neutral/ hydrophobic). Although this sequence was found in ~50 proteins in the Swiss-Prot data base, among these, there was only a single protein kinase, ASK1 (MAPKKS5), a component of the MAPK cascades (30). To determine whether ASK1 may be a direct target of SLK, COS cells were transfected with HA-SLK, with and without HA-ASK1. Lysates were immunoprecipitated with anti-HA antibody and subjected to an in vitro immune complex kinase assay. Whereas SLK and ASK1 independently autophosphorylated to a minor extent, cotransfection of SLK with ASK1 resulted in marked phosphorylation of ASK1 but not SLK (Fig. 4, A and B). Therefore, SLK most likely phosphorylates ASK1. Similar experiments were performed using HA-SLK 1–373 and HA-ASK1. SLK 1–373 contains the N-terminal catalytic domain but is missing the C-terminal domain and is reported to be more active than full-length SLK (9, 10). Similar to full-length SLK, SLK 1–373 was also able to phosphorylate ASK1 (Fig. 4, C and D). Although there was a minor degree of ASK1 autophosphorylation in these experiments, phosphorylation of SLK 1–373 was absent.

To determine whether SLK-induced phosphorylation of ASK1 leads to an increase in ASK1 activity, COS cells were transiently transfected with Myc-His-SLK and/or HA-ASK1. Lysates were immunoprecipitated with anti-HA antibody and subjected to an in vitro immune complex kinase assay. Cotransfection of SLK with ASK1 increased ASK1 activity (Fig. 4, E and F). Cotransfection of kinase-dead SLK (Myc-SLK K63R) and HA-ASK1 did not, however, enhance ASK1 activity (Fig. 4G). Finally, in cotransfection experiments, dephosphorylation of wild type SLK did not affect ASK1 phosphorylation (Fig. 4H), in keeping with the lack of phosphatase effect on SLK activity (Fig. 2D).
Role of SLK in Renal Ischemia-reperfusion Injury

SLK Activates the p38 MAPK Pathway—Activation of ASK1 is reported to lead to activation of MKK4/MKK7-JNK and MKK3/MKK6-p38 pathways (4). To determine whether SLK can signal via MAPK pathways, HA-SLK was overexpressed with or without HA-ASK1 in COS cells. Immunoblotting of lysates with phosphospecific antibodies demonstrated that there was some basal p38 phosphorylation in COS cells, but cotransfection of SLK and ASK1 increased p38 phosphorylation significantly (Fig. 5, A and B). In contrast, cotransfection of SLK and ASK1 did not affect phosphorylation of JNK (Fig. 5C). The reason for lack of JNK activation is unclear and perhaps may be related to weak expression of MKK4 and MKK7 in COS cells. Cotransfection of SLK and ASK1 did not affect phosphorylation of ERK (Fig. 5D).

Activation of SLK Enhances Apoptosis—These studies addressed the effect of SLK on apoptosis in the setting of ischemia-reperfusion injury in vitro (exposure of cells to chemical anoxia followed by re-exposure to glucose; anoxia/recovery). We employed cell lines (MDCK cells and/or GECs) that may be affected by renal ischemia-reperfusion injury in vivo. In keeping with previous results (16), anoxia/recovery increased SLK activity (Fig. 6A) and induced phosphorylation of p38 (Fig. 6B). The increase in p38 phosphorylation was 35% greater in cells that overexpressed SLK, as compared with Neo (control) cells (Fig. 6D). Anoxia/recovery induced phosphorylation of p38 (Fig. 6C and D) that was exacerbated in cells that stably overexpress SLK, as compared with Neo (control) cells (Fig. 6D). The next series of experiments addressed the pathways mediating apoptosis. Since SLK can lead to phosphorylation of p38 kinase (Fig. 5), we tested the effect of the p38 kinase inhibitor, SB203580, on apoptosis. In the presence of SB203580, the proapoptotic effect of anoxia/recovery was markedly attenuated both in Neo and SLK-transfected cells (Fig. 6D). Anoxia/recovery induced release of cytochrome c from mitochondria into the cytosol, and this effect was seen mainly in the SLK-transfected cells (Fig. 6E and F). Furthermore, anoxia/recovery induced increases in the activities of caspase-8 and -9, and these increases were amplified in the cells that overexpress SLK (Fig. 6G).
ER Stress Response Is Attenuated in Cells That Overexpress SLK

In the next series of experiments, we examined the effects of SLK on the induction of ER stress proteins Bip and/or Grp94 (31, 32). Tunicamycin is a nucleoside antibiotic that blocks N-linked glycosylation and is believed to cause an accumulation of misfolded proteins in the ER, thereby inducing ER stress (26). Ca\textsuperscript{2+} ionophores can induce ER stress via depletion of ER Ca\textsuperscript{2+} stores. Incubation of control MDCK cells with the Ca\textsuperscript{2+} ionophore, A23187, or with tunicamycin increased expression of Bip and Grp94 (Fig. 7, A and B). In contrast, although basal levels of Bip and Grp94 tended to be slightly higher in cells stably transfected with SLK, both A23187 and tunicamycin were not able to increase Bip and Grp94 expression in these cells (Fig. 7, A and B). By analogy, anoxia/recovery increased expression of Grp94 in control MDCK cells but not in MDCK cells that stably overexpress HA-SLK, C, representative immunoblot (lower panel, control; Ponceau staining); D, densitometric quantification. *, p < 0.03 versus untreated, n = 16.

Finally, in the presence of the caspase-9-directed inhibitor, LEDH-CHO, the proapoptotic effect of anoxia/recovery was abolished almost completely (Fig. 6F). Together, the results indicate that SLK exacerbates the proapoptotic effect of anoxia/recovery and that the mediators of apoptosis include p38 and caspase-9. Release of cytochrome c suggests that anoxia/recovery activates the mitochondrial apoptotic pathway.

ER Stress Response Is Attenuated in Cells That Overexpress SLK—We and others have demonstrated that ischemia-reperfusion may induce ER stress (26, 31, 32). In the next series of experiments, we examined the effects of SLK on the induction of ER stress proteins Bip and/or Grp94 (31, 32). Tunicamycin is a nucleoside antibiotic that blocks N-linked glycosylation and is believed to cause an accumulation of misfolded proteins in the ER, thereby inducing ER stress (26). Ca\textsuperscript{2+} ionophores can induce ER stress via depletion of ER Ca\textsuperscript{2+} stores. Incubation of control MDCK cells with the Ca\textsuperscript{2+} ionophore, A23187, or with tunicamycin increased expression of Bip and Grp94 (Fig. 7, A and B). In contrast, although basal levels of Bip and Grp94 tended to be slightly higher in cells stably transfected with SLK, both A23187 and tunicamycin were not able to increase Bip and Grp94 expression in these cells (Fig. 7, A and B). By analogy, anoxia/recovery increased expression of Grp94 in control MDCK cells but not in MDCK cells that stably overexpress SLK (Fig. 7, C and D). Anoxia/recovery induced a similar pattern in changes in Bip expression, although the difference was not statistically significant (data not shown).

A second aspect of the ER stress response is the induction of eIF2\textalpha phosphorylation, which leads to a global reduction in protein synthesis via inhibition of translation (32). Exposure of cells to anoxia/recovery...
resulted in rapid phosphorylation of eIF2α. Phosphorylation was, however, attenuated in cells that stably overexpress SLK (Fig. 8, A and B). Expression of eIF2α protein was not affected by anoxia/recovery (not shown). We were not able to detect phosphorylation of eIF2α in response to tunicamycin in both control and transfected cells. However, certain mRNAs are preferentially induced and translated after eIF2α becomes phosphorylated, including CHOP (GADD153) (32). Thus, we monitored induction of CHOP as an index of eIF2α phosphorylation. In keeping with the pattern of eIF2α phosphorylation induced by anoxia/recovery, incubation with tunicamycin increased CHOP expression in control cells, whereas the effect of tunicamycin was markedly attenuated in cells that stably overexpress SLK (Fig. 8, C and D). A third aspect of the ER stress response is activation of caspase-12, which is resident in the ER (31, 32). Caspase-12 cleavage was significantly greater in Neo GECs, as compared with GECs that stably overexpress SLK (Fig. 8, E and F).

The next set of experiments examined the proapoptotic effects of agents that activate stress pathways (including ER stress) together with SLK. Following anoxia/recovery, apoptosis increased markedly in SLK-overexpressing cells (Fig. 8G). Incubation with tunicamycin did not independently induce apoptosis, but when combined with anoxia/recovery, tunicamycin enhanced apoptosis (Fig. 8G). The apoptotic effect of tunicamycin plus anoxia/recovery was markedly exacerbated in cells that overexpress SLK (Fig. 8G). These results suggest that induction of the ER stress response has a protective effect (Neo cells), whereas an attenuated ER stress response (SLK-expressing cells) leads to increased apoptosis. The functional role of ER stress was confirmed by incubating cells with tunicamycin plus anoxia/recovery, in the presence or absence of salubrinal, a compound that blocks dephosphorylation of eIF2α, thus potentiating translation inhibition (33). In both Neo and SLK-expressing cells, apoptosis was almost entirely abolished with salubrinal (Fig. 8H), indicating that enhancement of eIF2α phosphorylation is cytoprotective.

**Effect of SLK Overexpression on the Cell Cycle**—The above studies demonstrate an important role for SLK in mediating apoptosis. In the next series of studies, we examined whether SLK expression could affect cell proliferation by modulating the phases of the cell cycle. By analogy to GECs (17), under standard culture conditions, increases in cell number for up to 72 h after plating were reduced in MDCK cells that overexpress SLK, as compared with control (Fig. 9A). Stable overexpression of SLK attenuated [3H]thymidine incorporation, which reflects DNA synthesis (S phase; Fig. 9B). Progression of cells through the cell cycle was analyzed by labeling of DNA with propidium iodide and monitoring DNA content by fluorescence-activated cell sorting (Fig. 9C). GECs that overexpress SLK showed a greater number of cells that underwent apoptosis (in keeping with the results in Fig. 6) and a lower number of cells both in G1 and G2/M phases, as compared with control cells. Together, the results suggest that in the context of SLK overexpression, a lower proportion of cells progresses through the cell cycle. However, based on these results, it was not possible to determine precisely in which cell cycle phase apoptosis occurs.

Stable overexpression of SLK did not affect expression of cyclin-dependent kinase inhibitors, p21 and p27, both in confluent and subconfluent cells (Fig. 9D). Cyclin D1 expression was also not significantly different in serum-deprived and serum-stimulated Neo GECs and GECs that overexpress SLK (Fig. 9E).

**DISCUSSION**

Expression and activity of the renal epithelial protein kinase, SLK, were increased during kidney development and following ischemia-reperfusion injury, and stable overexpression of SLK in cultured cells increased apoptosis and exacerbated cell death after cells were subjected to chemical anoxia/recovery (16). The present study has further characterized the activation and functional role of SLK. The N-terminal region of SLK contains a protein kinase domain, whereas the C-terminal domain contains a coiled-coil structure. Homodimerization of the C-terminal domain enhanced SLK activity (Fig. 2, A and B); however, the C-terminal domain of SLK did not heterodimerize with Mst1 (a related kinase that also contains a coiled-coil structure) and did not affect Mst1 activity (Fig. 3). In a previous study, it was shown that deletion of the C-terminal domain of SLK enhanced kinase activity, suggest-
Role of SLK in Renal Ischemia-reperfusion Injury

**FIGURE 9. Effect of SLK overexpression on the cell cycle.** MDCK cells were plated at 50,000 cells/well and were cultured for up to 72 h. Increases in cell number 48 and 72 h after plating were attenuated in MDCK cells that overexpress SLK, as compared with control (*, p < 0.05, n = 5). B, [3H]thymidine incorporation. GECs were plated at a density of 5,000 cells/well. After 24 or 48 h, GECs were pulsed with [3H]thymidine for 6 h. [3H]thymidine incorporation was significantly greater in Neo GECs, as compared with HA-SLK-transfected cells. p < 0.005, n = 4. C, cell cycle analysis. GECs, synchronized by contact inhibition and serum withdrawal, were plated for 24 h in serum-replete medium. Then DNA was labeled with propidium iodide, and DNA content was analyzed by fluorescence-activated cell sorting. The GECs that overexpress HA-SLK showed a greater number of cells that underwent apoptosis (subdiploid DNA content) and a lower number of cells in G1 and G2/M phases, as compared with Neo. *, GECs were treated as in A. D, expression of the cyclin-dependent kinase inhibitors, p21 and p27. Lysates of confluent and subconfluent Neo GECs and GECs that overexpress HA-SLK were immunoblotted with antibodies to p21 or p27. There were no apparent differences in expression between Neo and SLK-transfected GECs. Loading controls (Pontecorvo staining) are presented in D and E.

In the present study, phosphorylation or dephosphorylation of SLK was not associated with changes in kinase activity (Fig. 2, C and D). This result appears to be distinct from those reported in earlier studies, where treatment of SLK with phosphatases or phosphatase inhibitors altered autophosphorylation or kinase activity (9, 10, 34). The reason for this discrepancy will require further elucidation. Perhaps changes in phosphorylation regulate kinase activity only under certain physiological or experimental conditions, or they may be cell-specific.

Identification of substrates and downstream signals of group II–VIII GCKs has produced limited results so far. The present study demonstrates that SLK can induce phosphorylation of ASK1 (Fig. 4, A and B). ASK1 can be phosphorylated at distinct sites, some of which lead to an increase and others to a decrease in activity (35–38). The phosphorylation of ASK1 by SLK was associated with increased ASK1 activity (Fig. 4, E and F). Phosphorylation was induced by full-length SLK and by a truncated mutant that contained only the kinase domain (Fig. 4, C and D). Furthermore, ASK1 activation was dependent on the kinase activity of SLK, since a kinase-dead SLK mutant was unable to increase the activity of ASK1 (Fig. 4G). It should be noted that kinase-independent functions of SLK and other GCKs have been reported (2, 10). In the present study, we demonstrate that cotransfection of SLK and ASK1 stimulated phosphorylation of p38 kinase but not JNK nor ERK (Fig. 5). The amount of p38 phosphorylation was only slightly less than the amount induced by UV light. To our knowledge, this is the first demonstration of p38 pathway activation by SLK. This result is also consistent with known targets of ASK1, which include p38 (and JNK) (35, 36). In previous studies, conflicting results have appeared on JNK activation by SLK (i.e. one study demonstrated JNK activation (9, 10), but another study did not (17)). It should be noted that transfection of SLK alone (i.e. without ASK1 transfection) was not able to induce p38 phosphorylation effectively (Fig. 5, A and B), suggesting that protein kinases downstream of SLK may be rate-limiting. This may account for the discrepant results of the various studies of signaling pathways activated by SLK. Possibly, insufficient expression of some JNK pathway intermediary kinases in certain cells may preclude JNK activation by SLK. Apart from the MAPKs, polo-like kinase-1 was reported to be another substrate of SLK (34).

Relatively few stimuli have been shown to modulate activity of GCKs. In keeping with earlier results (16), we demonstrate that SLK activity was enhanced by anoxia/recovery (in vitro ischemia-reperfusion injury) and that overexpression of SLK enhanced anoxia/recovery-induced apoptosis (Fig. 6, A, C, and D). The protein kinase downstream of SLK (i.e. ASK1) is also generally regarded as a proapoptotic kinase (35, 36). By analogy to an earlier study (39), anoxia/recovery-induced apoptosis was associated with release of cytochrome c from mitochondria (40–42) as well as increases in caspase-8 and -9 activities, all of which were enhanced by SLK overexpression (Fig. 6, E–G). The mediators of apoptosis included p38 kinase as well as caspase-9 (Fig. 6, D and H) (40–42). Release of cytochrome c suggests involvement of the mitochondrial pathway in apoptosis, and the mitochondrial pathway could have been activated directly or secondary to ER stress (43, 44) (see below). Activation of caspase-8 may have been secondary to caspase-9 (27), or possibly, there may also be a role for death domains in anoxia/recovery-induced apoptosis. Our results are consistent with earlier studies, which showed that p38 can mediate apoptosis via the mitochondrial pathway, although the role of p38 in apoptosis may be cell type- and stimulus-dependent (45, 46). p38 activation has been demonstrated in ischemia-reperfusion in vivo (47). Moreover, translocation of Bax from the cytosol to the mitochondria has been demonstrated in anoxia/recovery in cell culture and was associated with cell death (39), although the amount of cell injury in this study appeared to be significantly greater, as compared with our model. Further studies will be required to delineate precisely how SLK and p38 induce apoptosis.

In the present (Fig. 9A) and earlier studies (17), we demonstrated that under standard culture conditions, increases in cell number were attenuated in GECs or MDCK cells that overexpress SLK, as compared with control cell lines (16). This result is consistent with a greater amount of apoptosis in the cells that overexpress SLK but might also be accounted for by anti-proliferative effects, including actions on cell cycle progression, particularly those that are mediated via MAPK pathways (48–50). Cell cycle analysis demonstrated that the cells stably transfected with SLK became apoptotic at a higher rate, as compared with control cells (Fig. 9C). Furthermore, a lower proportion of SLK-transfected cells was
in G₁ and G₂/M phases, as compared with control. These results are in keeping with the proapoptotic role of SLK demonstrated in Fig. 6 and earlier (16) and do not allow precise determination of the cell cycle phase where apoptosis occurs. A reduction of cells in S phase due to SLK overexpression (Fig. 9B) is also in keeping with a greater proportion of these cells undergoing apoptosis. Finally, we were not able to demonstrate effects of SLK on expression of the cyclin-dependent kinase inhibitor p21 and p27, as well as cyclin D1 (a G₁ phase cyclin) (Fig. 9, D and E). Together, these results suggest that the action of SLK is principally proapoptotic and that SLK does not appear to retard the rate of cell proliferation. Interestingly, an earlier study, which analyzed the expression/activity of SLK at various stages of the cell cycle suggested that SLK may actually facilitate progression through the cell cycle (34). In this regard, it should be noted that we were unable to generate GECs that stably overexpress a kinase-dead SLK mutant (results not shown), suggesting that perhaps SLK may indeed be essential for cell cycle progression. Although this would seem to be an apparent contradiction, the protein kinase, PKR, is actually reported to mediate cell survival and death at distinct time points (51).

Induction of the ER stress response (unfolded protein response) has been receiving increasing attention as a cellular mechanism of protection or recovery from injury (including ischemia-reperfusion injury), although prolonged or intense ER stress may lead to cell death (31, 32). The ER stress response is believed to be initiated by the presence of abnormal proteins in the lumen of the ER. Interaction of these proteins with Bip leads to the activation of ER sensors (IRE1, ATF6, and PERK). There are distinct aspects to the ER stress response, including induction of ER stress proteins that are cytoprotective (e.g. Bip and Grp94) via ATF6 and/or IRE1 (31, 32). Another aspect of the ER stress response is an increase in eIF2α phosphorylation via PERK. This pathway reduces general synthesis of proteins via inhibition of translation, which aims at keeping with the proapoptotic role of SLK demonstrated in Fig. 6 and injury (33) offers novel approaches to the therapy of acute renal failure.

**Role of SLK in Renal Ischemia-reperfusion Injury**

**Note Added in Proof—Recently, O’Reilly et al. (O’Reilly, P. G., Wagner, S., Franks, D. J., Cauli, K., Braway, E., Dissous, C., and Sabourin, L. A. (2005) J. Biol. Chem. 280, 42383–42390) demonstrated that SLK is required for progress through the G₂ phase of the cell cycle.**

**REFERENCES**

1. Kyriakis, J. M., and Avruch, J. (1996) BioEssays 18, 567–577
2. Kyriakis, J. M. (1999) J. Biol. Chem. 274, 5259–5262
3. Dan, I., Watanabe, N. M., and Kusumi, A. (2001) Trends Cell Biol. 11, 220–230
4. Kyriakis, J. M., and Avruch, J. (2001) Physiol. Rev. 81, 807–869
5. Kuramochi, S., Moriguchi, T., Kuida, K., Endo, J., Semba, K., Nishida, E., and Kara-suyama, H. (1997) J. Biol. Chem. 272, 22679–22684
6. Pombo, C. M., Bonventre, J. V., Molnar, A., Kyriakis, J., and Force, T. (1996) EMBO J. 15, 4537–4546
7. Pombo, C. M., Tsujita, T., Kyriakis, J. M., Bonventre, J. V., and Force, T. (1997) J. Biol. Chem. 272, 29372–29379
8. Graves, J. D., Gotoh, Y., Draves, K. E., Ambrose, D., Han, D. K., Wright, M., Chernoff, J., Clark, A. E., and Krebs, E. G. (1998) EMBO J. 17, 2224–2234
9. Sabourin, L. A., and Rudnicky, M. A. (1999) Oncogene 18, 7566–7575
10. Sabourin, L. A., Tarnai, K., Seale, P., Wagner, J., and Rudnicky, M. A. (2000) Mol. Cell. Biol. 20, 684–696
11. Sorokin, L., and Ekbloom, P. (1992) Kidney Int. 41, 657–664
12. Koseki, C., Herzlinger, D., and al-Awqati, Q. (1992) J. Clin. Cell Biol. 119, 1327–1333
13. Savill, J. (1999) Kidney Int. 56, 1216–1222
14. Liu, J., Takano, T., Papillon, J., Khadir, A., and Cybulsky, A. V. (2001) BioEssays 23, 79–80
15. Thadhani, R., Pascual, M., and Bonventre, J. V. (1996) N. Engl. J. Med. 334, 1448–1460
16. Cybulsky, A. V., Takano, T., Papillon, J., Khadir, A., Bijian, K., Chien, C. C., Alpers, C. E., and Rabb, H. (2004) Am. J. Physiol. Renal Physiol. 286, F16–F25
17. Yamada, E., Tsujiikawa, K., Itoh, S., Kameda, Y., Kohama, Y., and Yamamoto, H. (2000) Biochim. Biophys. Acta 1495, 250–262
18. Buse, P., Tran, S. H., Luther, E., Phu, P. T., Aponte, G. W., and Firestone, G. L. (1999) J. Biol. Chem. 274, 7253–7263
19. Srivaman, V. S., Wang, H., Nuovo, G. J., and Malbon, C. C. (1997) J. Clin. Invest. 99, 1478–1483
20. Atwater, J. A., Wisdom, R., and Verma, I. M. (1999) Annu. Rev. Genet. 21, 519–541
21. Bernstein, P., and Ross, J. (1989) Trends Biochem. Sci. 14, 373–377
22. Shaw, G., and Kamen, R. (1986) Cell 46, 659–667
23. Lupas, A., Van Dyke, M., and Stock, J. (1991) Science 252, 1162–1164
24. Coers, W., Reivinen, J., Miettinen, A., Huitema, S., Vos, J. T., Salant, D. J., and Weening, J. J. (1996) Exp. Nephrol. 4, 184–192
25. Cybulsky, A. V., Monge, J. C., Papillon, J., and McTavish, A. J. (1995) Am. J. Physiol. 269, F739–749
26. Cybulsky, A. V., Takano, T., Papillon, J., Khadir, A., Liu, J., and Peng, H. (2002) J. Biol. Chem. 277, 41342–41351
27. Bijian, K., Takano, T., Papillon, J., Khadir, A., and Cybulsky, A. V. (2004) J. Physiol. Renal Physiol. 286, F255–F266
28. Letunic, I., Copley, R. R., Schmidt, S., Ciccarelli, F. D., Doerks, T., Schultz, J., Ponting, C. P., and Bork, P. (2004) Nucleic Acids Res. 32, D142–D144
29. Brinkworth, R. I., Reinel, R. A., and Kobe, B. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 74–79
30. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saito, M., Moriguchi, T., Takagi, M., Matsuzawa, A., Nishitoh, H., and Ichijo, H. (2006) Cell Struct. Funct. 31, 23–29
31. Matsukawa, J., Matsuzawa, A., Takeh, K., and Ichijo, H. (2004) J. Biochem. (Tokyo) 136, 261–265
32. Galvan, V., Logvinova, A., Sperandio, S., Ichijo, H., and Bredesen, D. E. (2003) J. Biol. Chem. 278, 13325–13332
Role of SLK in Renal Ischemia-reperfusion Injury

38. Yuan, Z. Q., Feldman, R. I., Sussman, G. E., Coppola, D., Nicosia, S. V., and Cheng, J. Q. (2003) J. Biol. Chem. 278, 23432–23440
39. Saikumar, P., Dong, Z., Patel, Y., Hall, K., Hopfer, U., Weinberg, J. M., and Venkat-achalam, M. A. (1998) Oncogene 17, 3401–3415
40. Green, D. R., and Reed, J. C. (1998) Science 281, 1309–1312
41. Kroemer, G., and Reed, J. C. (2000) Nat. Med. 6, 513–519
42. Reed, J. C. (2000) Am. J. Pathol. 157, 1415–1430
43. Demaurex, N., and Distelhorst, C. (2003) Science 300, 65–67
44. Zong, W. X., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, Q. C., Yuan, J., and Thompson, C. B. (2003) J. Cell Biol. 162, 59–69
45. Van Laethem, A., Van Kelst, S., Lippens, S., Declercq, W., Vandenabeele, P., Janssens, S., Vandenheede, J. R., Garmyn, M., and Agostinis, P. (2004) FASEB J. 18, 1946–1948
46. Song, J. J., and Lee, Y. J. (2004) J. Cell. Biochem. 92, 1257–1270
47. Park, K. M., Chen, A., and Bonventre, J. V. (2001) J. Biol. Chem. 276, 11870–11876
48. Lavoie, J. N., I’Allemain, G., Brunet, A., Muller, R., and Pouyssegur, J. (1996) J. Biol. Chem. 271, 20608–20616
49. Wilkinson, M. G., and Millar, J. B. (2000) FASEB J. 14, 2147–2157
50. Sherr, C. I., and Roberts, J. M. (2004) Genes Dev. 18, 2699–2711
51. Donze, O., Deng, J., Curran, J., Sladek, R., Picard, D., and Sonenberg, N. (2004) EMBO J. 23, 564–571
52. Kebache, S., Cardin, E., Nguyen, D. T., Chevet, E., and Larose, L. (2004) J. Biol. Chem. 279, 9662–9671