Renal expression and activity of the germinal center kinase SK2

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Cybulsky, Andrey V., Tomoko Takano, Joan Papillon, Abdelkrim Khadir, Krikor Bijian, Chu-Chun Chien, Charles E. Alpers, and Hamid Rabb. Renal expression and activity of the germinal center kinase SK2. Am J Physiol Renal Physiol 286: F16–F25, 2004. First published September 9, 2003; 10.1152/ajprenal.00144.2003.—Rat fetal kidney mRNA was analyzed by RT-PCR to identify protein kinases. This screening demonstrated expression of a protein kinase consistent with SK2, a group II germinal center kinase and homolog of human Ste20-like kinase (SLK). SK2 mRNA, protein expression, and kinase activity were increased in rat fetal kidney homogenates (embryonic days 17–21) compared with adult controls. In adult kidneys subjected to cross-clamping of the renal artery, followed by reperfusion, SK2 mRNA, protein expression, and kinase activity were increased compared with untreated contralateral controls. By immunohistochemistry, SK2 expression was evident mainly in the cytoplasm of tubular epithelial cells in fetal and adult kidneys. There was also some expression in developing and mature podocytes, but staining of the interstitium was negative. In cultured renal tubular epithelial cells, SK2 kinase activity was increased after incubation with serum, or after exposure to chemical anoxia plus reexposure to glucose. Stable overexpression of SLK reduced cell proliferation and increased apoptosis and exacerbated apoptosis and necrosis after chemical anoxia plus reexposure to glucose. Thus SK2 is a renal epithelial protein kinase whose expression and activity are increased during development and recovery from acute renal failure, where tubular epithelial regeneration may recapitulate developmental processes. The actions of SK2 appear to be antiproliferative and may facilitate cell injury.

EXTRACELLULAR SIGNALS are transmitted to nuclear or cytoplasmic effectors through a series of cytoplasmic serine/threonine protein kinases, known as the mitogen-activated protein kinases (MAPK). There are several parallel MAPK pathways, including the extracellular signal-regulated kinase (ERK) pathway, typically activated by growth factors, and the “stress-activated” c-Jun NH2-terminal kinase (JNK) and the p38 kinase pathways, activated by diverse stimuli, including cytokines (e.g., tumor necrosis factor), heat shock, hyperosmolality, etc. (17, 22, 27, 40). ERKs have multiple potential targets, and their biological roles may include mediation of cell proliferation, survival, differentiation, or migration (27). The biological roles of JNKs and p38 kinase pathways include withdrawal from the cell cycle and mediation of apoptosis or initiation of cell repair (17, 22). Germinal center kinases (GCK) are mammalian homologs of the yeast MAPK kinase kinase, Ste20 (21, 22). GCKs share homology in their NH2-terminal catalytic domains but not in their COOH-terminal regulatory domains (21). Group I GCKs include GCK, GCK-related kinase, hemopoietic progenitor kinase, Nck-interacting kinase, and others (21). These kinases interact with MAPK kinase kinase kinase 1 and activate JNK. Some group I GCKs are effectors of tumor necrosis factor. Group II GCKs include mammalian Ste20-like (MST) 1–3, Ste20-like oxidant stress-activated kinase (SOK) 1 (30, 31), lymphocyte-oriented kinase (LOK; see Ref. 20), and human Ste20-like kinase (SLK; see Ref. 48) and its mouse (36, 37) and rat homolog, SK2 (unpublished). There is relatively little known about the regulation of group II GCKs (21). Some of the group II GCKs may be activated posttranslationally by stress (e.g., SOK1 is activated by oxidants, ischemia, or ATP depletion; see Ref. 31). Structural features of group II GCKs (e.g., presence of a coiled-coil domain) suggest that activation might involve dimerization and autophosphorylation. Generally, group II GCKs appear not to activate any of the known MAPK pathways (21).

Receptors that signal via MAPKs play an important role in kidney development. Interaction of the ureteric bud with the metanephric mesenchyme at embryonic gestational day (E) 11–12 in the mouse and rat or week 4–5 in humans initiates metanephric kidney development (13, 45). After the initial interaction, the ureteric bud branches in the mesenchyme. Eventually, the ureteric bud forms the collecting system, whereas its outgrowths will develop into the collecting ducts of the kidneys. In parallel, the mesenchymal cells adjacent to the ureteric bud are induced to aggregate and convert into epithelium, which in later gestation will proliferate and differentiate into the epithelial component of tubules and glomeruli. The appearance of endothelial and mesangial cells in the developing kidney is stimulated by differentiating epithelium, although the precise origin of these cells has not been determined conclusively (16). Several growth factors and growth factor receptor tyrosine kinases may participate in nephrogenesis. At the onset of kidney development, c-ret is expressed by the ureteric bud, and kidney development is impaired in c-ret knockout mice (39). Organ culture studies suggest that insulin-like growth factor-I (IGF-I), transforming growth factor (TGF)-α, and their corresponding receptors may play a role in tubulogenesis (13). Platelet-derived growth factor receptor-β mediates differentiation of mesangial cells and the glomerular tuft (44). Endothelial cell-specific receptor tyrosine kinases, including Tie-2, are expressed in the kidney, as well as several growth factors with angiogenic potential that act via receptor tyrosine kinases (16). Epidermal growth factor (EGF) receptor
expression and kinase activity are increased in rat kidneys in late gestation (E16–21; see Ref. 7). Embryonic kidneys in organ culture produce TGF-α, and anti-TGF-α antibody inhibits tubulogenesis in vitro (13). Some strains of EGF receptor knockout mice show developmental abnormalities in collecting duct epithelium and develop renal failure perinatally (47). Among the MAPK pathways, ERK and p38 are strongly expressed in developing kidney (14), and inhibition of ERK or p38 pathways may disrupt nephrogenesis and kidney growth (11, 14).

In acute renal failure (so-called “acute tubular necrosis”), tubular epithelial cells undergo sublethal and lethal injury. Ischemic acute renal failure is characterized by ATP depletion, acidosis, and metabolite accumulation, and, when blood flow is restored (“reperfusion”), injury may occur via production of reactive oxygen species (24, 46). It has been proposed (3) that, after acute ischemia, tubular epithelial regeneration may proceed along steps similar to developmental nephrogenesis. Thus viable cells would dedifferentiate, proliferate, and then redifferentiate into a mature, functional phenotype (3, 24, 28, 46). Moreover, growth factor-regulated kinases, e.g., ERK, might promote recovery via induction of cell proliferation or survival and counteract ischemia-induced (stress-activated) kinase pathways, e.g., JNK (28). For example, the EGF receptor may mediate tubular regeneration (15). Moreover, administration of exogenous EGF, IGF-I, or hepatocyte growth factor to experimental animals accelerates recovery of tubular function (24, 28). Studies directly addressing the activation of MAPK cascades in acute renal failure are limited. JNK is activated transiently just after the ischemic insult, during reperfusion (29), and JNK inhibition improves recovery (28). By analogy, in a cell culture model that recapitulates the ATP depletion and oxidant stress of ischemia (i.e., chemical anoxia induced by inhibition of the electron transport chain and glycolysis), re-exposure to glucose activated JNK. In contrast, chemical anoxia (via oxidant stress) directly activated SOK1 (31).

During identification of fetal kidney receptor tyrosine kinases (18), we cloned a serine-threonine kinase partial cDNA that was compatible with the group II GCK, SLK/SK2. In the present study, we characterize the expression and activation of SLK/SK2 in kidney development and ischemic acute renal failure. We demonstrate that SK2 expression and kinase activity are increased in the developing kidney and in kidneys recovering from acute renal failure. Studies in cultured epithelial cells suggest that SLK/SK2 may antagonize cell proliferation and facilitate necrotic and apoptotic cell death.

MATERIALS AND METHODS

Materials. Tissue culture and molecular biology reagents were obtained from Invitrogen Life Technologies (Burlington, ON). Electrophoresis and immunoblotting reagents were from Bio-Rad Laboratories (Mississauga, ON). Myelin basic protein and glutathione-agarose were purchased from Sigma (St. Louis, MO). [α-32P]dCTP (3,000 Ci/mmol) and [γ-32P]ATP (3,000 Ci/mmol) were purchased from PerkinElmer Canada (Woodbridge, ON). Hoechst H-33342 dye was purchased from Calbiochem (San Diego, CA). Mouse anti-hemagglutinin antigen epitope tag (HA) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Male and pregnant female Sprague-Dawley rats and New Zealand White male rabbits were purchased from Charles River Canada (St. Constant, PQ). The mammalian expression vector pEF-HA-SLK was kindly provided by Dr. Kazutake Tsujikawa, Osaka University (Osaka, Japan). Rat SK2 cDNA was kindly provided by Dr. Yasuo Fukami, Kobe University (Kobe, Japan).

Ischemic acute renal failure. Ischemic acute renal failure was induced in male rats weighing 150–200 g, as described previously (33). Briefly, rats were anesthetized with pentobarbital sodium injection (50 mg/kg). A midline abdominal incision performed under sterile conditions was followed by clamping of the right kidney with a vascular clip. The clip was released at 30 min, and reperfusion was observed. Temperature was maintained constant, and warm saline (5% of body wt) was administered. After awakening, animals had free access to food and water. Experimental techniques were in accordance with National Institutes of Health guidelines for animal experimentation.

Preparation of kidney RNA and protein. Kidneys were dissected from fetal rats at E16–21 or from adult male rats. Fetal kidneys were then pooled, and total RNA was isolated from rat fetal and adult kidneys, using TRIzol reagent, according to the manufacturer’s instructions. Postnuclear supernatants containing cytosol and membrane fractions were prepared as described earlier (7). The recovery and distribution of proteins in membrane preparations have been described previously, and there were no significant differences in these parameters between fetal and adult kidneys (7).

RT-PCR and Northern blotting. RT-PCR (34 cycles) was carried out using total RNA from rat fetal or adult kidney as template and primers for SK2, LOK, or β-actin (control; see Ref. 18). PCR primers were designed based on distinct cDNA sequences of SK2 and LOK catalytic domains. The sequences were as follows: SK2 forward, CTGTGCGCTGGAATGT; SK2 reverse, CTCCTTTGAAATTGCTG; LOK forward, CGAGAGAGAAGATCTCCG; and LOK reverse, GAGTTCGTTTTGGAGTTTCA. The PCR cycle was 45 s at 94°C (denaturation), 45 s at 55°C (annealing), and 1 min at 72°C (elongation), with a final 10-min elongation at 72°C. The PCR products of SK2 (NH2-terminal region) and LOK were subcloned into the pBluescript II or pCRII vectors (Invitrogen) and were sequenced. SK2 or LOK cDNA inserts were excised with appropriate restriction enzymes, labeled with [α-32P]dCTP, and used as probes for Northern blotting, as described previously (10). Densitometry of multiple Northern blots was performed using NIH Image software.

Preparation of anti-SK2 antiserum. cDNA encoding the 350 COOH-terminal amino acids of rat SK2 was subcloned into the vector pGEX-5X-2 (Amersham Pharmacia Biotech). A GST-SK2 (COOH-terminal domain) fusion protein was produced and purified using glutathione-agarose, according to the manufacturer’s instructions. Two rabbits were immunized with the fusion protein to produce anti-SK2 antiserum. An IgG fraction of the antiserum was prepared using a HiTrap rProtein A column (Amersham Pharmacia Biotech), according to the manufacturer’s instructions.

Immunoblotting, immunoprecipitation, and complex kinase assays. Methods for immunoblotting and immunoprecipitation were described previously (18). For IgG complex kinase assays (9), 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 PMSF, 1 mM Na2VO4, 3 mM EDTA, and 3 mM EGTA, pH 7.5 (4°C). Proteins were immunoprecipitated with rabbit anti-SK2 antibody (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 mM HEPES, 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). After a 5-min incubation at 30°C, the reaction was terminated by the addition of Laemmli buffer, and the mixture was subjected to separate gel electrophoresis. Densitometry of immunoblots or autoradiograms was performed using NIH Image software.

Cell culture and transfection. Madin-Darby canine kidney (MDCK) cells and COS-1 cells were cultured in DMEM-10% FCS,
Table 1. Homology of cloned fetal kidney serine/threonine protein kinase

<table>
<thead>
<tr>
<th>Percent Homology</th>
<th>Protein Kinase</th>
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<tbody>
<tr>
<td>&gt;90</td>
<td>Mouse LOK (D89728)</td>
</tr>
<tr>
<td>80</td>
<td>SK2/SLK</td>
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<tr>
<td></td>
<td>Rat protein kinase (AB003357)</td>
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<td></td>
<td>Human KIAA0204 (D686959)</td>
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<tr>
<td></td>
<td>Human SLK (AB002804)</td>
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<tr>
<td></td>
<td>Guinea pig serine/threonine kinase (D88425)</td>
</tr>
<tr>
<td></td>
<td>Mouse protein kinase (L10016)</td>
</tr>
<tr>
<td></td>
<td>Mouse Ste20-related kinase SMK (AF112855)</td>
</tr>
<tr>
<td>64</td>
<td>Mouse Rab-11-SAPK (U50595)</td>
</tr>
<tr>
<td>56</td>
<td>Human HPK1 (U66464)</td>
</tr>
<tr>
<td>52</td>
<td>Human MST1 (U18297)</td>
</tr>
<tr>
<td>52</td>
<td>Human SOK1 (X93925)</td>
</tr>
<tr>
<td>48</td>
<td>Yeast Ste20 (AAA35039)</td>
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The predicted amino acid sequences of protein kinase catalytic domains VI-VIII were compared. Accession numbers are given in parentheses. See text for definitions.

Rat glomerular epithelial cell (GEC) culture and characterization have been published previously (10, 32). GEC were cultured in K1 medium, and studies were done with cells between passages 8 and 60. Stable transfection of GEC with HA-SLK cDNA was performed by the calcium phosphate technique, as described previously for cytosolic phospholipase A2 and other proteins (8, 10). After primary selection with G418, stably transfected clones were selected by immunoblotting with anti-HA antibody.

**Measurement of proliferation, apoptosis, and cytolysis.** Changes in cell number, reflecting cell proliferation, were determined by trypsinization of adherent cells, followed by visual counting of cells in a hemacytometer. To quantitate apoptosis, adherent cells were stained with Hoechst H-33342 dye (1 μg/ml) for 10 min at 37°C (without fixation; see Ref. 23). After being washed, cells were stained with propidium iodide (1 μg/ml) to exclude necrotic or late-apoptotic cells (23). Nuclei of apoptotic cells were stained brightly with H-33342 dye and stained negatively with propidium iodide. Cells were photographed using a Nikon Diaphot immunofluorescence microscope and Nikon Coolpix 995 digital camera, and the number of H-33342-positive/propidium iodide-negative cells was quantified by visual counting.

Cytolysis (necrotic cell death) was determined by measuring release of lactate dehydrogenase (LDH), similarly to the method described previously (10, 32). Specific release of LDH was calculated as (E - C)/(100 - C), where E represents the percentage of total LDH released in cell supernatants in incubations under experimental conditions, and C is the percentage of total LDH released in cell supernatants in incubations under control conditions.

**Immunohistochemistry.** Tissues were fixed in formalin or methyl Carnoy’s solution (60% methanol, 30% chloroform, and 10% acetic acid) and processed and embedded in paraffin according to conventional techniques (6, 41). Immunohistochemistry was performed on 4-μm sections of paraffin-embedded tissues following a standard avidin-biotin complex (ABC) method. Briefly, sections were deparaffinized in xylene and rehydrated with graded ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide, and the samples were then rinsed in PBS. For reduction of background labeling, the sections were blocked by using the avidin-biotin blocking kit (Vector Laboratories) per the manufacturer’s instruction and normal goat serum for 30 min. The sections were incubated overnight at 4°C with rabbit anti-SK2 IgG diluted 1:100 in PBS containing 1% BSA and 5% nonfat dry milk. After being washed in PBS, the sections were incubated sequentially with biotinylated goat anti-rabbit IgG (Vector Laboratories) and the ABC-Elite reagent (Vector Laboratories). Finally, 3,3′-diaminobenzidine (with nickel chloride enhancement) was used as the chromogen, and sections were counterstained with methyl green, dehydrated, and covered with a coverslip. For all samples, a negative antibody control consisted of substitution of the primary antibody with normal rabbit IgG.

**Statistics.** Data are presented as means ± SE. The t-statistic was used to determine significant differences between two groups. One-way ANOVA 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**

The serine/threonine protein kinase SK2 is expressed in rat fetal and adult kidney. To identify receptor tyrosine kinases expressed in the developing kidney, rat fetal kidney mRNA was analyzed by RT-PCR, using degenerate oligonucleotide primers to highly conserved regions of receptor tyrosine kinases (protein kinase catalytic domains VI-VIII). This screening demonstrated expression of various receptor tyrosine kinase mRNAs, published earlier (18). However, on two occasions, the screening yielded one nonreceptor tyrosine kinase sequence, with the predicted amino acid sequence IHRDLK-AGVNLMVTLEGDIDRALFVSAKNLKTQLQKDSFFIGTPYWMAPE. Comparison of this sequence with other sequences in a gene database revealed that the sequence showed greatest similarity to group II GCKs, in particular LOK and rat SK2/SLK (Ste20-like kinases; Table 1). Using RT-PCR and primers specific to either LOK or SK2, it was demonstrated that SK2 mRNA is expressed in rat fetal kidney (E17, E19, and E21) and rat adult kidney (Fig. 1). In contrast, there was no significant (or only trivial) renal expression of LOK (Fig. 1).

Northern blot analysis confirmed the absence of renal LOK mRNA expression (data not shown) and the presence of SK2 (Fig. 2). Based on these results, we proceeded to investigate the expression, activity, and potential functional role of SK2 in the kidney. The rat and human forms of the protein kinase are structurally very similar, and, in the studies described below, we refer to the rat protein kinase as SK2 and to its human homolog as SLK.

**Renal expression of SK2 mRNA.** Northern blot analysis demonstrated the presence of SK2 mRNA in rat kidney (Fig. 2A). At least two mRNAs (7.0 and 5.0 kbp) were evident, and expression of both types was significantly higher in fetal kidney (E17, E19, and E21) compared with normal adult kidney (a representative Northern blot is shown in Fig. 2A, and densitometric quantification in Fig. 2C). This result suggested that SK2 is a developmentally regulated protein kinase.
It is believed that, after ischemic renal injury, renal tubular epithelial cells may recover by dedifferentiating, proliferating, and then redifferentiating into mature tubular epithelium. Therefore, recovery from ischemic injury may resemble the process of renal development. Changes in SK2 mRNA expression were studied in a model of unilateral renal ischemia-reperfusion injury involving 30-min occlusion of the renal artery. SK2 mRNA was increased 12, 24, and 48 h after ischemia (Fig. 2, B and C).

**SK2 protein expression and kinase activity.** To enable studies of SK2 protein expression, rabbit antisera were produced against the COOH-terminal domain of SK2. The antisera identified a protein of ~200 kDa in COS cells transfected with HA-SLK (Fig. 3A). This size is in keeping with an earlier study (36, 48) but is greater than the calculated molecular mass. The same ~200-kDa band was also present in anti-SK2 antibody immunoblots in untransfected COS cells, indicating endogenous SLK (endogenous SLK and HA-SLK are of similar size). RT-PCR demonstrated SLK mRNA in untransfected COS cells (data not shown), in keeping with endogenous expression of SLK protein. Anti-SK2 antibody specifically immunoprecipitated an ~200-kDa protein in rat GEC (Fig. 3B).

Changes in SK2 protein expression were studied by immunoblotting. By analogy to mRNA, expression of SK2 protein was significantly higher in fetal kidney (E18–E20) compared with normal adult kidney (Fig. 4, A and E). In unilateral ischemia-reperfusion injury, SK2 protein expression increased in the postischemic kidneys compared with contralateral control (Fig. 4, B and E). The expression of SK2 protein in kidneys of two sham-operated rats was 83% of levels in contralateral control kidneys, suggesting that ischemia had no major systemic consequences on SK2 expression. SK2 kinase activity was assessed by specifically immunoprecipitating SK2 and monitoring phosphorylation of myelin basic protein. Reaction time was brief (5 min) to reduce the possibility of autoactivation, reported with group II GCKs (21). Kinase activity was significantly greater in fetal kidneys compared with normal adult kidney (Fig. 4, C and D). The increases in SK2 kinase activity were, in part, secondary to the increases in protein levels, but increases in kinase activity were ~25% higher than the changes in protein expression, suggesting that there may also have been increases in the catalytic activity of SK2.
RENAL EXPRESSION AND ACTIVITY OF SK2

During screening of rat fetal kidney mRNA for protein tyrosine kinases, we discovered an mRNA consistent with a group II GCK, including SK2/SLK. Further studies showed autophosphorylation of SK2 could be demonstrated in fetal kidneys (Fig. 4C) but was not detected consistently in adult kidneys. SK2 kinase activity was significantly greater in normal adult rat kidneys subjected to ischemia-reperfusion injury (Fig. 4, D and E).

Localization of SK2. SK2 protein was localized in rat kidney by immunohistochemistry (Fig. 5). In both fetal (E20) and normal adult kidneys, there was strong staining of renal proximal and distal tubular epithelial cells. Fainter or more patchy staining was present in GECs. There was no definitive staining of vascular structures. In fetal kidney, positive staining was also evident in some immature renal interstitial cells. Staining of the renal interstitium was negative in adult kidneys.

Functional role of SK2. To address the potential functional roles of SK2, it was necessary to establish models in cultured cell lines that are representative of cells where SK2 expression was demonstrated in vivo. First, we examined whether SK2 was expressed and activated in MDCK cells, a renal tubule epithelial cell line. Under basal conditions, there was expression of SK2 protein, and there appeared to be some kinase activity (Fig. 6). Renal development features cell proliferation and apoptosis, processes that are typically regulated by growth factors. Accordingly, we assessed the effect of FCS (which is enriched in growth factors) on changes in SK2. Addition of FCS to serum-deprived MDCK cells resulted in an increase in SK2 activity at 24 h, although not at 4 h (Fig. 6A). There were no significant serum-induced changes in SK2 protein expression (Fig. 6B). To model ischemia-reperfusion injury in culture, MDCK cells were exposed to chemical anoxia and recovery in complete (glucose-replete) culture medium (see legend to Fig. 6). Anoxia plus recovery (24 h) stimulated a rise in SK2 kinase activity (Fig. 6C). There was no effect of anoxia/recovery on SK2 protein expression (Fig. 6D).

In a second series of experiments, GEC were transfected with HA-SLK cDNA, and three clones of cells that stably overexpress HA-SLK protein were selected for further study (Fig. 7). Overexpression of HA-SLK attenuated the increase in cell number on the third day after plating (statistically significant reduction in two of the three clones; Fig. 8A). The reduction in the number of GEC was most likely the result of a reduction in the rate of proliferation and, at least in part, the result of an increase in apoptosis, although increased apoptosis reached statistical significance in only one clone (Fig. 8B).

In contrast to SK2, transfection of GEC with another protein kinase, i.e., a constitutively active MAP/ERK kinase (26 and unpublished observations), which activates ERK, tended to increase the rate of proliferation, compared with neo cells (Fig. 8C). Thus the reduced rate of proliferation in cells overexpressing HA-SLK is unlikely to have been because of a nonspecific effect of protein kinase transfection/overexpression. It is important to note that the cytotoxic or proapoptotic properties of SLK may have prevented production of clones with a high level of SLK overexpression. In GEC exposed to chemical anoxia plus reexposure to glucose (24 h), HA-SLK overexpression increased cell death, including LDH release (Fig. 9A) and apoptosis (Fig. 9B). The increase in LDH release was consistently greater than apoptosis, indicating that at least a portion of LDH release was probably the result of cytolysis/necrosis. It should be noted that changes in cytolysis were evident only after the recovery period, i.e., there was no specific LDH release detected just after exposure of neo and HA-SLK clones to chemical anoxia (data not shown). Thus the actions of SK2 appear to be antiproliferative and may facilitate cell injury.

DISCUSSION

Fig. 4. SK2 protein expression and kinase activity. A: SK2 protein expression in rat fetal (E18, E19, and E20) and normal adult kidneys (immunoblot; equal amounts of total proteins were loaded in each lane). SK2 migrates at ~200 kDa. There were no apparent differences among E18, E19, and E20. B: SK2 protein expression in adult rat kidneys subjected to ischemia and reperfusion (24 h; Isch) and in contralateral control (Ctrl) kidneys (immunoblot). C: SK2 kinase activity in rat fetal and adult kidneys. Kinase activity was measured by immunoprecipitation of fetal (E18, E19, and E20) or adult kidney homogenates with anti-SK2 antisera and incubation of immune complexes with myelin basic protein (MBP) and [γ-32P]ATP (5 min, 30°C). The autoradiogram shows MBP phosphorylation, as well as autophosphorylation of SK2. D: SK2 kinase activity in renal ischemia-reperfusion injury. SK2 kinase activity was measured in postischemic and contralateral control adult rat kidneys. E18 fetal kidney is shown for comparison. E: densitometric quantification of protein expression and kinase activity (MBP phosphorylation; arbitrary units). *P < 0.001 and **P < 0.0001, fetal (E18–E20, n = 6) vs. adult (n = 2). ***P < 0.0005 and ****P < 0.0025, Isch (n = 4) vs. Ctrl (n = 4).
that expression of SK2 mRNA and protein, as well as kinase activity, was increased in rat fetal kidney homogenates (E17–E21) compared with adult control kidneys (Figs. 2 and 4). In adult kidneys subjected to 30 min of cross-clamping of the renal artery, followed by reperfusion, SK2 mRNA and protein expression and kinase activity were increased compared with untreated contralateral control kidneys (Figs. 2 and 4). By immunohistochemistry, SK2 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 GEC (podocytes; Fig. 5). Thus SK2 is a renal epithelial protein kinase in which expression and activity are increased during development and recovery from acute renal failure, where injured tubular epithelial cells may regenerate by recapitulating developmental processes. In cell culture, incubation of epithelial cells with serum, or exposure to chemical anoxia, followed by reexposure to glucose (recovery), increased SK2 activity (Fig. 6). Finally, overexpression of SK2 in epithelial cells reduced proliferation under normal culture conditions (Fig. 8), probably, at least in part, because of apoptosis, and in the setting of anoxia-exacerbated apoptosis and probably necrosis (Fig. 9).

Some biochemical and functional aspects of SLK were characterized previously (36, 37, 48). Analysis of the complete predicted amino acid sequence of SLK/SK2 demonstrates that the kinase has 1,204 amino acids. There is an NH2-terminal serine/threonine kinase catalytic domain, an ATP-binding site at K63, and a COOH-terminal regulatory domain. SLK is most similar to the protein kinase LOK (46% identical in full-length, 77% identical in the kinase domain; see Ref. 20). Analysis of mRNA expression indicates that SLK is expressed ubiquitously (1, 36, 48). Overexpression of SLK did not lead to activation of ERK, or p38 pathways, but conflicting results have been published on JNK activation, i.e., one study failed to demonstrate JNK activation, whereas activation was observed in another (36, 37, 48). Transient overexpression of SLK induced apoptosis (36, 37), and in these studies the authors were unable to generate stably transfected cell lines, since overexpression appeared to be cytotoxic. EGF, anisomycin, and hyperosmolality did not stimulate SLK activity in transfected COS-7 cells, and cotransfection with constitutively active Ras, Rac, or cell division cycle (Cdc)-42 also did not enhance SLK activity (48). These characteristics are generally in keeping with other group II GCKs (21).
The GCKs and p21-activated kinases are homologous to Ste20 of Saccharomyces cerevisiae (21). There are at least 11 mammalian kinases related to GCK. All GCKs possess NH2-terminal kinase domains and extensive COOH-terminal regulatory domains, but GCKs do not possess Cdc42/Rac interactive binding motifs and do not bind Rho GTPases. Group I GCKs interact with MAPK kinase kinase 1; consequently, they activate the JNK pathway but not ERK or p38 pathways. Several group I GCKs are activated by tumor necrosis factor.

Fig. 6. Effect of serum and chemical anoxia/glucose reexposure (recovery; A/R) on SK2 protein and kinase activity in Madin-Darby canine kidney (MDCK) cells. A and B: MDCK cells were cultured in serum-poor medium (DMEM-0.5% FCS) for 24 h. Next, cells were cultured in serum-poor or serum-replete medium (DMEM-10% FCS) for 4 or 24 h. A: SK2 kinase activity was measured as in Fig. 4. In this autoradiogram, the serum-poor sample is at 4 h, but there was no significant change in SK2 activity in serum-poor medium over a 24-h period. B: protein expression was determined by immunoprecipitation with anti-SK2 antiserum (+), or nonimmune serum in controls (−), and immunoblotting of immune complexes with anti-SK2 antiserum. SK2 migrates at ~200 kDa. C and D: MDCK cells were incubated in glucose-free buffer for 40 min (37°C). Incubation was continued in either glucose-free buffer (Control) or glucose-free buffer plus 10 mM 2-deoxyglucose and 10 μM antimycin A (chemical anoxia) for 90 min. Cells were then incubated in glucose-replete medium (DMEM-10% FCS) for 4 or 24 h (recovery). SK2 kinase activity (C) and protein expression (D) were determined as above. E: densitometric quantification of kinase activity (MBP phosphorylation; arbitrary units). *P < 0.02, serum-poor (SP) vs. serum (S), 24 h (n = 6). **P = 0.05, anoxia/recovery, 24 h vs. control (although the differences did not reach statistical significance if the 4-h anoxia/recovery time point was included in the analysis; n = 8).

Fig. 7. Stable transfection of GEC with HA-SLK cDNA. Lysates of transfected GEC were immunoblotted with anti-HA antibody. Three clones that stably express HA-SLK protein (16, 73, and 310) and neo GEC are presented. Untransfected COS cells or COS cells transiently transfected with HA-SLK are presented for comparison. The bottom band in the immunoblot is probably nonspecific.

The GCKs and p21-activated kinases are homologous to Ste20 of Saccharomyces cerevisiae (21). There are at least 11 mammalian kinases related to GCK. All GCKs possess NH2-terminal kinase domains and extensive COOH-terminal regulatory domains, but GCKs do not possess Cdc42/Rac interactive binding motifs and do not bind Rho GTPases. Group I GCKs interact with MAPK kinase kinase 1; consequently, they activate the JNK pathway but not ERK or p38 pathways. Several group I GCKs are activated by tumor necrosis factor.
Their biology has been reviewed recently (21). **Group II** GCKs do not fit into the well-defined MAPK pathways. In addition to SLK/SK2, the **group II** GCK family includes MST1–3, SOK1, and LOK. **Group II** GCKs are expressed ubiquitously, with the exception of LOK, which is expressed in lymphocytes. These kinases do not activate ERK, JNK, or p38 pathways except for MST1 and perhaps SLK, which have been reported to activate JNK (12).

**Regulation of group II GCK activity is poorly understood** (21). Protein kinases are generally believed to be regulated by phosphorylation. By analogy to SK2, regulation of kinase activity via changes in mRNA/protein expression (e.g., transcriptional regulation) has been reported for other protein kinases (5, 43), although not for other GCKs, and the understanding of the role of transcriptional regulation is limited. GCKs may possess significant basal activity when immunoprecipitated from endogenous sources or when overexpressed (21, 48). However, MST1 and SOK1 can also be activated in vivo by various stresses, e.g., heat shock, arsenite, staurosporine, ischemic injury, or ATP depletion, and an increase in cytosolic Ca\(^{2+}\) concentration is required for SOK1 activation (30, 31). In vitro, MST1 can be activated by phosphatase 2A, while activities of SOK1 and SLK appear to be dependent on autophosphorylation (30, 36). Interestingly, SLK is reported to be a target for caspase-3, such that proteolytic cleavage of SLK may release and activate the NH\(_2\)-terminal kinase domain from the COOH-terminal domain and increase kinase activity (37). However, we did not detect any proteolytic fragments of SK2 in our study. SLK (and other GCKs) have a coiled-coil structure in their COOH-terminal regulatory domain, and proteins with the coiled-coil structure are known to oligomerize by means of this structure (25). For example, SOK1 and MST1 spontaneously homodimerize in vivo. Because SLK is constitutively active in transfected COS-7 cells (48), it is reasonable to propose that increased expression of SK2/SLK in the developing kidney or after ischemic injury may facilitate interaction of SK2/SLK with itself or other coiled-coil proteins and enhance kinase activity. Similar to other GCKs, SLK also contains four potential Pro/Glu/Ser/Thr-rich (PEST) sequences between the NH\(_2\)-terminal kinase domain and the COOH-terminal coiled-coil region (48). The PEST motif is related to rapid degradation of proteins (34, 35); consequently, kinase activity may be downregulated in part by a short half-life resulting from the PEST domain. Finally, the 3' region of SLK mRNA (48) contains at least two polyadenylation signals (AAUAAA), and six so-called stability motifs (AUUUA), suggesting that SLK mRNA is unstable (2, 4, 42). Thus mRNA stability may also regulate SK2 protein expression and, secondarily, kinase activity. Further studies to address these possibilities are in progress.

Kidney development is dependent on the presence of growth factors, activation of growth factor receptors, and interaction of cells with extracellular matrixes (45). During development of renal structures, there is a proliferation of renal cells and apoptosis (19, 38), 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 (24, 46). Ischemia typically injures the renal tubular epithelial cells and is characterized by ATP depletion and other metabolic derangements, whereas reperfusion (on restoration of blood flow) is associated with production of reactive oxygen species. The cells that survive the ischemic insult may undergo a process resembling development, which includes dedifferentiation, reentry in the cell cycle, and proliferation to replace the dead cells. In cultured cells, chemical anoxia and glucose reexposure recapitulate the ATP depletion and production of reactive oxygen species seen in ischemia. To address the functional role of SK2 in kidney development, in the present study we stimulated epithelial cells with growth factors (serum). In addition, chemical anoxia plus reexposure to glucose was employed to model ischemic acute renal failure and reperfusion. Interestingly, both incubation conditions produced increases in SK2 activity, in keeping with results observed in vivo, although the changes observed in vivo included increases.

**Fig. 9. Effect of HA-SLK overexpression on anoxic GEC injury.** A: GEC were subjected to chemical anoxia plus glucose reexposure (recovery; 24 h), similar to the MDCK cells presented in Fig. 6. Cytolysis [lactate dehydrogenase (LDH) release] was enhanced significantly by HA-SLK overexpression. B: representative photomicrographs of two GEC clones subjected to chemical anoxia plus glucose reexposure and then stained with Hoechst H-33342. Arrows point to apoptotic cells, which feature condensed nuclei that stain more brightly compared with nuclei of normal cells that are larger and faintly stained. C: HA-SLK overexpression increased apoptosis in two clones significantly and induced an upward trend in one clone. \( *P<0.0002, \ \ddagger P<0.045, \ \ddagger\ddagger P<0.02 \) vs. neo 16. A: \( n=3–5 \); B: \( n=13–14 \).
in activity and protein expression, whereas increased activity was prevalent in cell culture models. The reason for this discrepancy will require further study. SLK was stably overexpressed in epithelial cells, and these cells were exposed to serum or to chemical anoxia followed by glucose reexposure. With the recognition that overexpression experiments have to be interpreted with a degree of caution, these studies demonstrated that the actions of SK2 under normal culture conditions are antiproliferative and that this action may be at least in part the result of increased apoptosis. After anoxia, SK2 facilitates necrotic cell death, as well as apoptosis. Thus activation of SK2 may attenuate cell proliferation during kidney development or recovery from ischemic injury. Furthermore, activation of SK2 may actually exacerbate injury or delay recovery. It should be noted that use of kinase inactive mutants of group II GCKs is generally not practical, since these mutants retain some biological effects of the wild-type kinases and can activate downstream targets (21).

The localization of SK2 in GEC together with the functional properties described above suggest that SK2 may be involved in development of podocytes, or in the pathogenesis of podocyte-specific glomerulopathies, e.g., membranous nephropathy. This aspect will require additional investigation. Further studies will also be required to define the antiproliferative or procytotoxic mechanisms of SK2 precisely and to verify the functional role in vivo. Understanding the role of GCKs in the kidney may eventually lead to new therapeutic strategies for renal dysplasia and ischemic acute renal failure.

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