Identification of a novel kidney-specific gene downregulated in acute ischemic renal failure

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ACUTE RENAL FAILURE (ARF) is a debilitating illness for which there is limited effective therapy (37). ARF can be induced by multiple factors including renal ischemic injury, nephrotoxic drugs or by other glomerular and tubular-interstitial diseases. ARF is associated with acute cellular damage in the proximal and distal tubules followed by tubular degeneration and necrosis, leading to decreased glomerular permeability, tubular leakage and impaired renal functions (1, 2, 24, 27, 38).

Experimentally, renal artery occlusion (43) and toxic compounds such as mercuric chloride and folic acid (25) have been used in rodents to induce acute renal failure. Renal artery occlusion in rat consistently produces acute renal failure as evidenced by a rapid decrease of glomerular filtration rate (GFR) and oliguria. Although the precise mechanisms for cell damage and necrosis are not clear, ischemic shock is thought to play an important role in the initial insult to proximal tubule cells (4, 23). The site of injury is believed to be at the S3 segment of the proximal tubule and the ascending limb of the loop of Henle. Loss of epithelial cell polarity is an important early hallmark (26, 29). Necrosis and apoptosis in epithelial cells are generally recognized to play important roles in the rapid loss of glomerular filtration (22).

A number of peptide hormones have been implicated in the etiology of the onset of ARF (12, 14). In particular the role of vasoconstrictors such as endothelin (ET) has been extensively studied (5, 10). Infusion of ET-1 causes reduction of renal blood blow (RBF) and GFR, while administration of ET-1 antagonists help restore renal function in animal models of ARF (10). Other peptide hormones such as atrial natriuretic peptide (ANP) and IGF-1 have also been shown to be effective in improving renal function in animal models (15, 18, 40). In clinical trials, however, neither IGF-1 nor ANP has been shown to be beneficial (3, 14, 37). Thus far, no effective drug therapy exists for ARF.

We have undertaken a systematic effort to isolate and characterize kidney-specific genes. By using a recently available PCR-select technique (7), we have isolated a pool of genes that are expressed predominantly in renal tissue and have discovered one of these genes is highly regulated in a rat model of acute ischemic renal failure. In this paper, we describe the isolation and characterization of this gene, termed kidney-specific protein 32 (KSP32).

MATERIALS AND METHODS

Materials. A PCR-select subtraction kit was obtained from Clontech (Palo Alto, CA). Nylon membrane (Biotran) was purchased from ICN Biotechnologies (Costa Mesa, CA). [α-32P]dATP (3,000 Ci/mmol) was purchased from NEN-Du-
Pont (Boston, MA). Oligo(dT) agarose was obtained from Pharmacia Biotech (Piscataway, NJ). Routine molecular cloning and sequence analyses were performed as previously described (35). Reagents for subcloning and sequencing were purchased from Promega (Madison, WI). PCR reagents were obtained from Perkin-Elmer (Norwalk, CT). Random priming labeling kits were obtained from Promega. Mice poly(A)$^+$ mRNA blot was purchased from Clontech. For in vivo experiments, SB-209670 was synthesized at SmithKline Beecham Pharmaceuticals (King of Prussia, PA) and was prepared in 5% dextrose (vehicle).

Computer programs used to analyze the protein sequences include BLASTN, BLASTp, and Laser-gene-DNA star programs.

**RNA isolation.** mRNA from wild-type Sprague-Dawley rat (SD) tissues (brain, heart, liver, lung, intestine, spleen, pancreas, and kidney) was isolated as described (35). Briefly, 1–5 g of tissue were homogenized in 20 ml of homogenization buffer (4 M guanidium thiocyanate, 25 mM Na citrate, 0.5% sarcosyl, and 0.1 M beta-mercaptoethanol) by using a Polytron (Kinematica) homogenizer for 30 s at the highest speed (~10,000 rpm). Homogenized tissue samples were then centrifuged at 5,000 g for 20 min to clear the debris. Supernatants were then carefully added to a 9-ml Beckman ultracentrifuge tube (no. 361623) that contained 4.5 ml of CsCl solution (5.7 M CsCl, 25 mM Na citrate, 0.1 M EDTA). Tubes were sealed and spun at 50,000 rpm (Ti 70.1 rotor, Beckman) for 24 h. RNAs were pelleted at the bottom of the tube and were harvested by using 70% ethanol wash. RNAs were then dissolved in diethyl pyrocarbonate-treated water and frozen at -80°C until use.

**mRNA isolation.** mRNA was isolated by using oligo(dT) agarose column as described (35). Generally, 1–2 mg of RNA from different tissues were used to isolate up to 20 μg of mRNA.

**PCR-select procedure.** PCR-select was performed as described in the manufacturer’s instructions (Clontech). Briefly, 5 μg of mRNA were used for reverse transcription. Five micrograms of kidney mRNA were used to generate renal tester cDNA. For the rat kidney driver pool (see RESULTS), 1.25 μg of mRNA each from liver, brain, muscle, and intestine were used. For both driver and testers, a total of 5 μg mRNA were used to synthesize first and second cDNA strands. The double-stranded cDNA was subsequently digested with Dpn II restriction enzyme to produce the driver cDNA. Kidney cDNA driver and pooled four-tissue cDNA drivers were then ligated to either adaptor 1 or adaptor 2 according to the manufacturer’s instruction. The pooled four-tissue cDNA driver was then used as countersubtraction to...

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**Fig. 1. Cloning of KSP32 from rat and human.** Full-length nucleic acid sequence for human (A) and rat (B) KSP32 mRNA. Translated protein sequence was listed in situ under the nucleotides. Potential sites for protein kinase C were marked as *, and sites for casein kinase II were highlighted as #. The GenBank accession number for human and rat KSP32 are AF230095 and AF230096, respectively. C: comparisons of KSP32s and homologous proteins in GenBank. Three most conserved regions (A, B, and C) were underlined. GenBank accession numbers for the Caenorhabditis elegans, Pinus radiata, and Arabidopsis thaliana were as shown in the figure. D: hydrophilicity, antigenicity, and surface probability of KSP32 protein were analyzed by DNA star program and were plotted.
verify the subtraction efficiency. After ligation with adaptor 1 and adaptor 2, two rounds of hybridization were performed by using excess unligated driver cDNA. Two rounds of PCR (P1 and P2) were performed to amplify the subtracted and enriched cDNA species. After PCR amplification, the mixtures were analyzed on 2% agarose gels by hybridization to cDNA probes from kidney or the four-pooled tissues. If enrichment was observed, the PCR mixture was ligated to either pPCR2.1 or pPCR-TOPO (Invitrogen, Carlsbad, CA), and colonies were screened with cDNA probes.

Rapid amplification of cDNA ends (RACE 5' and 3') to clone the full coding sequence of KSP32 was performed by using the cDNA templates (Marathon cDNA) purchased from Clontech. Internal primers were coupled with the 5' or 3' marathon primers to PCR the KSP32 cDNA. PCR products were cloned into TA cloning vector (Invitrogen) and sequenced.

Cloning of PCR fragments was performed by using PCR2.1 or PCR-TOPO vectors according to the manufacturer's instructions (Invitrogen). After transformation and plating of bacteria onto IPTG-X-Gal dishes, white colonies were selected and restreaked onto two duplicated ampicillin dishes, with one dish having grided nylon membrane. After overnight growth, both dishes contained colonies, and nylon membranes were lifted to perform bacterial colony hybridization as described (35). Positive hybridizing clones were picked from the duplicate dish, and their DNA was isolated and sequenced.

Homology searches were performed by using BLASTN, BLASTX, FASTN, and FASTP (NCBI BLAST server, National Institutes of Health, Bethesda, MD).

Northern analyses. Ten micrograms of RNA from various rat tissues were separated on 1% agarose formaldehyde gels and transferred to a nylon membrane. After cross-linking, the nylon membrane was hybridized with random primed 32P-labeled cDNA probes as described (35).

Animal preparation. All procedures were approved by the Institutional Animal Care and Use Committee (Smith-Kline Beecham Pharmaceuticals) and were in accordance with National Institute of Health Guidelines for the care and use of animals. Young (6-wk-old) male SD were obtained from Charles River Laboratories (Wilmington, MA). After quarantine, the right kidney was removed from 50% of the rats under isoflurane anesthesia. A 2-wk recovery period was allowed for the hypertrophy of the remaining kidney. Vascular catheters were implanted into the abdominal aorta and inferior vena cava 2–4 days before the experiments. The rats were housed at all times in a light-controlled room with a 12:12-h light-dark cycle and were allowed ad libitum access to food and water.

Experimental protocol. For the occlusion of the renal artery, the rats were anesthetized with pentobarbital (40 mg/kg iv). The left renal artery was exposed through a small flank incision and occluded for 50 min with a nontraumatic clamp. Body temperature was maintained at 37°C throughout the occlusion. Three rats underwent the surgical procedure without the occlusion of the artery and were used as sham. The incision was closed after the removal of the clamp, and the rats were returned to their home cage to recover. The rats were divided into two experimental groups.

Group 1: Short-term recovery after ischemia (6 rats). The left kidney was removed under ether anesthesia at 5 or 24 h after ischemia. Then the rats were killed with an overdose of pentobarbital.

Group 2: Prolonged recovery after ischemia (34 rats). One day after ischemia, the rats underwent 3-h iv infusion of either vehicle, or the mixed ET-1 receptor antagonist SB-209670 (30 μg·kg⁻¹·min⁻¹). The sham animals received only...
vehicle. The kidney was harvested and the rats were killed in three groups, on the 2nd, 4th, or 14th day after ischemia.

For all animal experiments, a blood sample (500 µl) was taken from all rats before the ischemic challenge and at indicated time points of experiments (5 and 24 h for group 1 and 24, 48, and 96 h, and 14 days for group 2 rats). The plasma was separated and analyzed for electrolytes and creatinine concentration. The latter was used as an index of renal glomerular filtration.

Dissection of kidney and separation of glomeruli and tubular cell fractions. Glomeruli and tubular cell fractions were prepared using a sieving technique (8, 9). Briefly, kidneys were removed from male SD (300–350 g, Charles River) and immediately placed in cold dissection buffer containing (in mM) 137 NaCl, 5 KCl, 1 CaCl2, 1.2 MgSO4, 5.5 glucose, 10 sodium acetate, 3 lactate, and 20 HEPES (pH 7.4). The kidneys were decapsulated and bisected. Cortex, pelvis, inner medulla, and outer medulla portions were excised by using razor blades. For glomeruli preparation, the cortex and outer medulla were minced into a fine paste with a razor blade and pressed through a 230-µm stainless steel sieve. The resulting material was suspended in HEPES buffer and forced through sieves of decreasing pore sizes of 140, 104, and 75 µm. The glomeruli, which were trapped on the 75-µm sieve, were washed from the sieve and pelleted at 1,500 g for 5 min. The flow through and materials retained at the larger sieve were also collected and RNA was prepared from them. The purity of the glomeruli was checked by light microscopy.

In situ RNA analyses. In situ RNA hybridization analyses were performed as in Laping et al. (21) with minor modifications: 15-µm sections of frozen kidney were mounted on RNase-free slides by using a cryostat. Sections were fixed in 4% paraformaldehyde for 30 min. Sections were then treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, dehydrated in an ethanol series, and incubated with 0.3 ng/µl 21-mer 35S-labeled KSP32 cRNA probe in hybridization solution [50% formamide, 4× standard sodium citrate (SSC), 5× Denhardt’s, 1% SDS, and 250 µg/ml yeast transfer RNA] overnight at 50°C under parafilm slips. Parafilm was removed in 4×SSC and nonspecific hybridizations were removed by incubating slides in 0.025 mg/ml RNase A (in 0.5 M NaCl and 0.05 M phosphate buffer) at 37°C for 30 min. The wash was performed at 60°C for 30 min under paraffin slips. Paraform was removed in 4× SSC and nonspecific hybridizations were removed by incubating slides in 0.025 mg/ml RNase A (in 0.5 M NaCl and 0.05 M phosphate buffer) at 37°C for 30 min. The wash was performed at 60°C for 30 min under paraffin slips. Paraform was removed in 4× SSC and nonspecific hybridizations were removed by incubating slides in 0.025 mg/ml RNase A (in 0.5 M NaCl and 0.05 M phosphate buffer) at 37°C for 30 min. The wash was performed at 60°C for 30 min.
Developed slides were counterstained with hematoxylin and eosin.

Antibody generation and Western analyses. A glutathione-S-transferase (GST)-KSP32 fusion protein was constructed by using PCR to insert KSP32 cDNA fragment into pGEX-2tk vector in frame. The transformed *Escherichia coli* (BL21) was harvested and used to generate and purify soluble fusion GST-KSP32 protein according to instructions (Pharmacia Biotech, Piscataway, NJ). Purified GST-KSP32 was injected into rabbit for polyclonal antibody. Serum bleed from the rabbits was used for the Western blot by using 1:1,000 dilutions. Enterochromaffin-like (ECL) Western blot procedure was used to visualize KSP32 protein (Amersham, Arlington Height, IL).

Total soluble kidney protein was prepared by standard procedures (35). Briefly, whole kidney was homogenized in rat intrapulmonary arteries lysis buffer containing 1% Triton-100, 1% Na deoxycholate, 0.1% SDS, 10 mM Tris–HCl, pH 8.0, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 mM iodoacetamide. After centrifugation at 10,000 *g* at 4°C for 1 h, supernatants were collected and protein concentrations were measured by using Bio-Rad DC reagents (Bio-Rad, Hercules, CA). Fifty micrograms of total kidney proteins were separated on a SDS-NU-PAGE gel (4–12% gradient; Novex, San Diego, CA) and transferred to polyvinyl difluoride member for Western blot.

**RESULTS**

**Molecular cloning of KSP32.** A systematic search for rat kidney-specific genes was performed by using a PCR-select subtraction cloning strategy (17). Approximately 200 clones were obtained by using this procedure. One of these clones, R86, did not reveal any significant homology to other rat or human proteins in the database. Northern blot analyses indicated that the mRNA corresponding to R86 was highly kidney specific. To obtain full-length cDNA clones for R86, 5' and 3' RACE was performed to obtain a full coding sequence. This gene was subsequently named KSP32 since it is expressed almost exclusively in the kidney (see below). To obtain a human cDNA clone, a human kidney cDNA library was screened with the rat cDNA probe at lower stringency and the clones obtained were sequenced. A full-length cDNA clone for human KSP32 was verified by sequencing.

Rat and human KSP32 is a 285-amino acid protein with high homology to each other (96% similarity) (Fig. 1, A and B); however, searching the database with KSP32 revealed only four proteins with limited homology. One is an open reading frame from the *Caenorhabditis elegans* genome (accession no. AAB92032), identified by the genome sequencing project. Two putative proteins from *Arabidopsis thaliana* were also identified (accession no. CAB38955 and CAC62136). In addition, a gene isolated from *Pinus radiata* (Monterey pine) that was implicated in embryonic genesis of the pine (accession no. AAC05150) also showed significant similarity to KSP32 (Fig. 1C). The homology of KSP32 and these four proteins are concentrated in the amino acid range 30–280, with a 60–70% similarity to each other. The *C. elegans* protein has a longer NH₂-terminal extension that is not found in other proteins including KSP32 (Fig. 1C). The *C. elegans* protein also has a
much shorter COOH terminus. Blocks of highly conserved regions are clearly identifiable (regions A, B and C in Fig. 1C). All of the three regions contain aromatic amino acid residues (residues W, H, F, or Y) in the center of the region. It is currently unclear what the significance of these structure homologies are, however, the strong evolutionary conservation suggests a fundamental biological function for this protein.

There is no apparent motif for secretion at the NH$_2$ terminus (leader sequence) in the KSP32 protein. Five potential phosphorylation sites for casein kinase II and two potential phosphorylation sites for protein kinase C...
Fig. 3. In situ RNA hybridization of KSP32 in rat kidney. A: schematic diagram of kidney cross sections and renal tubule arrangement around medullary rays. Adapted from Knepper et al. (19, 20). B: 35S exposure of in situ RNA hybridization for KSP32 expression in rat kidney longitudinal and cross sections. Both sense and anti-sense KSP32 cRNA were labeled with 35S-UTP and hybridized with fixed kidney sections on slides (see MATERIALS AND METHODS). Exposure time is ~3 days. C: Microscopic pictures of KSP32 mRNA in situ expression. Phase contrast images are a, c, e, g, and corresponding dark field images are b, d, f, and h, respectively. a, b, c, and d are cross-sectional pictures, while e and g are longitudinal sections. g and h are higher magnifications, where glomeruli (G) are visible. Inner medulla (IM), outer medulla (OM), and medullary rays (MR) (arrows) were marked. The position of proximal tubules (PT) and collecting tubules (CT) (e) were also labeled. Emulsion exposure time was ~30 days.
(Fig. 1A and B) were identified. Hydrophilicity and other analyses (Fig. 1D) revealed that the protein was highly acidic with an isoelectric point of 5.63. KSP32 protein contains 29 aspartic acid residues and 9 glutamate residues. No nuclear localization signal sequence (NLS) is apparent in the sequence. Long stretches of hydrophilic regions are easily identifiable. These results suggest KSP32 is a highly charged, acidic cytoplasmic protein.

Expression of KSP32 mRNA in tissues. Tissue distribution of KSP32 in both human and rat was examined by Northern blot analyses. In both human and rat tissues, KSP32 expression was found predominantly in kidney (Fig. 2A). Longer exposure of the X-ray film revealed much lower levels (~1–5% of kidney) of KSP32 mRNA in rat liver (Fig. 2A). The mRNA corresponding to KSP32 is ~1.4 kb in length.

By using a polyclonal antibody made against KSP32, we detected abundant protein with the molecular mass of 32 kDa only in kidney tissues, and it was absent in various cell lines (Fig. 2B) and other tissues examined...
The molecular mass of KSP32 protein agreed well with the predicted molecular mass (34 kDa) derived from cDNA sequences.

To determine the anatomic location of KSP32 expression in the kidney, we fractionated medulla, cortex, glomeruli, and tubules by using physical dissection and sizing the cells through meshes (9). RNA from whole kidney, pelvis, medulla, cortex, isolated glomeruli, and tubular cells was analyzed for KSP32 mRNA expression. As shown in Fig. 2C, KSP32 mRNA was abundant.

Fig. 4. Expression of KSP32 mRNA and protein in acute renal failure (ARF). A: renal function measurements of ARF. Group 1 (6 rats) were for short-term ischemia experiment (5 and 24 h), and group 2 (34 rats) were for longer-term ischemia experiments with or without endothelin (ET) antagonist (48 h, 96 h, and 14 days). Plasma creatinine was measured as a standard procedure as described in MATERIALS AND METHODS. Surgery was performed on two groups of rats (groups 1 and group 2, MATERIALS AND METHODS), and plasma was withdrawn from rats at indicated time points for creatinine measurements. The number of rat creatinine measurements were indicated. Creatinine levels at 5 and 24 h were significantly higher than basal level (P < 0.01). The decrease in creatinine levels at 72 and 96 h in SB-209670-treated rats was statistically significant compared with vehicle-treated rats (P < 0.05). Compared with Sham animals, the dramatic elevation of creatinine levels from 24 to 96 h in both vehicle- and SB-209670-treated rats was highly statistically significant (P < 0.01). B: Northern blot and Western blot analyses of KSP32 in short-term ARF. Ten micrograms of total RNA from rat kidneys 5 or 24 h after renal injury were separated on 1.2% agarose gel and analyzed by KSP32 Northern blot. Kidney extract (50 µg total soluble kidney protein) was also analyzed in Western blot for KSP32 expression. C: Northern blot and Western blot analyses of KSP32 mRNA 48 h, 96 h, and 14 days postinjury with or without ET antagonist (SB-209670). S, sham animals. D, drug (SB-209670)-treated animals. V, vehicle-treated animals. K-cadherin (K-Cad) is a recently identified gene whose expression is highly restricted to kidney (42). Ethidium bromide stain was used as a control for RNA loading. For Western blot analysis, 50 µg of total kidney protein were analyzed.

(data not shown).
in cortex, medulla/cortex mix, and tubular fraction (Mesh 3/4 FT), while glomeruli, pelvis, and inner medulla demonstrated little expression.

We have also sought to identify primary kidney cells or kidney cell lines that express KSP32 in cell culture condition. To our surprise, none of the cells we have examined, including primary human mesangial cells, proximal tubule epithelial cells, cortical epithelial cells as well as several kidney epithelial cell lines (NRK, HK2), expressed significant amount of KSP32 mRNA or protein (data not shown). Furthermore, when kidney slices were cultured in a organ culture, KSP32 mRNA expression was rapidly lost in culture condition (data not shown). These results may suggest that certain in vivo factors such as cell-cell interactions, oxygen state, and micronutrient environment may be required for KSP32 expression.

**In situ analyses of KSP32 expression.** $^{35}$S-labeled anti-sense KSP32 RNA probe gave a very strong signal on whole kidney sections while the sense strand did not give any signals (Fig. 3B). The expression of KSP32 mRNA was predominant at the corticomedullary junction in both longitudinal and cross sections (Fig. 3B) and in particular a raylike expression pattern is clearly visible in cross section. The signal was totally absent in the inner medulla and was sporadic in the outer cortex region. Prominent expression was localized in the inner stripe of the outer medulla as well as the outer stripe and medullary rays. This expression pattern agrees very well with the Northern blot analyses performed with the fractionated kidney (Fig. 3, B and C). Microscopic examination of KSP32 expression revealed the KSP32 was likely to be expressed in proximal or distal tubules of the kidney in the outer medullar region and extending into the cortex (Fig. 3C). Strong signals were observed in tubules in medullary rays, inner and outer stripes (Fig. 3C, a and b). The KSP32 signal abruptly disappeared at the inner medulla (Fig. 3C, c and d), forming a clear boundary. In longitudinal sections, a strong signal was also visible in tubules around the bladder lumen (Fig. 3C, e and f). In contrast, little or no signal was observed in glomeruli (Fig. 3C, g and h). Presently it is not completely clear whether the proximal tubule or the ascending loop of Henley was responsible for the signal. Further studies will be needed to address this issue.

**Regulation of KSP32 expression in ischemic-induced renal failure.** Because proximal and distal tubule were severely impacted in ARF, we investigated whether KSP32 expression was affected in a rat model of ischemic ARF. Northern blot analyses (Fig. 4B) revealed that KSP32 mRNA was rapidly and dramatically downregulated in uninephrectomized rats upon ischemic renal injury. The kidney KSP32 mRNA was $\sim$50% of normal levels 5 h following ischemic injury and reached 5% of normal levels after 24 h. Serum creatinine levels were increased at 5 h (from 0.5 to 1.9 mg/dl) and at 24 h (to 4.5 mg/dl) (Fig. 4A). These results suggest that the decline of KSP32 mRNA closely reflected the decrease of renal function in ARF.

To extend these results further, we expanded the time points following renal injury and examined the
effect of an endothelin antagonist, SB-209670, which has been shown to be effective in ischemia-induced renal failure models (11). As shown in Fig. 4C, a dramatic reduction in KSP32 mRNA expression was observed 48 h following injury in both vehicle- and SB-209670-treated rats. The KSP32 mRNA remained repressed in vehicle-treated rats at 96 h and only recovered after 14 days. In contrast, in SB-209670-treated rats a significant amount of KSP32 mRNA was observed at 96 h and full recovery of KSP32 mRNA level was observed after 14 days (Fig. 4C). As a control, the expression of another kidney-specific gene, K-cadherin (42), was evaluated and its expression was not altered significantly under these conditions. Actin mRNA expression also remained unchanged (data not shown). Plasma creatinine measurements confirmed the impairment of renal function and the beneficial effect of SB-209670 (Fig. 4A). These data suggest that the expression of KSP32 mRNA not only closely reflected the rapid decline in renal function but also closely paralleled the recovery phase of ARF.

In addition to mRNA, KSP32 protein was also monitored by Western blot analysis in both the acute and prolonged ischemia experiments. At the 5-h time point, very little decrease in KSP32 protein was observed in ischemic kidney protein extract (lanes 3–5, Fig. 4B). This is perhaps due to the stability of KSP32 protein. At 24 h, however, two of the three rats had dramatically reduced KSP32 protein (Fig. 4B). Furthermore, the expression of KSP32 protein at 48 h has all been dramatically reduced (lanes 3–9, Fig. 4C) in both the ET antagonist- and vehicle-treated-kidneys. Two ET antagonist-treated rats at 96 h retained higher levels of KSP32 compared with vehicle-treated samples (lanes 10 and 11 vs. 12–15, Fig. 4C). At 14 days, most of the rats recovered from renal failure and KSP32 protein levels also recovered. Thus the level of KSP32 protein paralleled the decline of KSP32 mRNA in ischemic renal injury.

In situ analyses of rat KSP32 mRNA expression after ischemic renal injury. Consistent with the Northern blot analyses, whole-mount exposure of kidney sections following hybridization to 35S-labeled KSP32 rRNA probe indicated that KSP32 expression was dramatically reduced upon renal injury (Fig. 5A). A slight but consistent recovery of KSP32 mRNA expression was observed in SB-209670-, but not in vehicle-treated animals at 96 h. At 14 days, both SB-209670- and vehicle-treated rats demonstrated significant expression of KSP32 mRNA; however, the expression of KSP32 mRNA in SB-209670-treated rats was more intense around the medulla/cortex boundary and its formation resembled that of normal kidney (Fig. 5A). In contrast, vehicle-treated rats had a scattered-expression renal pattern for KSP32 mRNA (Fig. 5A).

When examined microscopically, KSP32 mRNA expression in recovering kidneys was mostly below the detection level at 48 h in vehicle- and SB-209670-treated rats (Fig. 5B, panel a and b). Low but visible expression, however, was detected in SB-209670-treated rats at 96 h while no expression was detected in the vehicle group. At these earlier time points, the morphology of the kidney clearly demonstrated that the cortex and medullary portions were severely damaged as was evidenced by low cellularity, large void spaces, and generalized disorganization (Fig. 5B, a and b). At the 14-day time point, a sporadic expression, especially in the cortex, was visible in the vehicle-treated group (Fig. 5B, c and d) while in the SB-209670-treated group expression of KSP32 was much more pronounced with the expression level approaching that of uninjured kidneys (Fig. 5B, e, f, g, and h). The ray-like formation of KSP32 signal was clearly visible in the SB-209670-treated group but was absent in the vehicle-treated group (Fig. 5B, e and f). In addition, the KSP32 mRNA was expressed in a much wider area at 14 days than observed in uninjured kidneys with prominent KSP32 signal extending into the cortex (Fig. 5B, g and h).

**DISCUSSION**

In this paper we describe the cloning and characterization of a novel renal-specific gene, termed KSP32, and its regulation in a rat model of ischemic renal failure. The KSP32 gene encodes a protein of ~32 kDa with little apparent homology to known mammalian...
proteins. Four proteins found in *C. elegans*, *P. radiata*, and *A. thaliana*, however, demonstrate a high degree of homology to KSP32. In addition, extensive homology (96% similarity) between the rat and human KSP32 suggests an important evolutionarily conserved function. Our data also indicate that the expression of KSP32 was highly restricted to the kidney. Only a limited amount (<1% of the amount found in kidney) of KSP32 was detected in the liver. The expression profile suggests a unique role for the protein in kidney function. In situ hybridization analyses revealed that the site of expression for KSP32 was primarily in the boundary of the cortex and outer medulla, especially within the medullary rays. Finally the expression of KSP32 was dramatically downregulated in a rat model of ischemic renal failure induced by renal arterial occlusion.

Gene regulation during ARF has been extensively documented (28, 31–33). Many genes have been found to be elevated, while only a limited number are suppressed (32). Two examples of genes that undergo downregulation in ARF are prepro-EGF mRNA (34) and the Tamm-Horsfall protein (THP) (33, 39). The kinetics of the reduction for EGF and THP mRNA is similar to KSP32: it begins immediately following injury and reaches a basal level at ~24 h. Several other proteins, BMP-7 (36) and IGFBP binding proteins-3 and -5 (16), have also been shown to be modestly
reduced. It is unclear what role such reduction of the gene expression plays in renal failure. One hypothesis (33) is that these genes are downregulated to permit a new round of cell replication events to repair the damage. Thus the reduction of these genes is needed to “dedifferentiate” cells and allow them to reenter the cell cycle, eventually leading to repair of the tubules and restoration of renal function. Another possibility is that these genes, especially KSP32, are required for maintaining the cell polarity of the differentiated renal tubule cells in the proximal region in the outer medulla and cortex. Damage in these areas leads to cell necrosis and/or cell apoptosis. Thus the loss of KSP32 mRNA expression may be a direct reflection of proximal tubular cell damage, either by necrosis or by apoptosis in situ.

The hypothesis that KSP32 mRNA is decreased due to the loss of tubular cells is consistent with its expression pattern in kidney. In situ analyses of KSP32 mRNA distribution revealed that the KSP32 is primarily found in the outer medulla and outlining a ray-like formation into cortex. This formation bears a remarkable resemblance to the medullary rays that reside in the outer stripe of the outer medulla. This is the region that contains most of the proximal and distal tubules. These tubules are severely damaged in the early phase of ARF (4, 23). Since the expression of KSP32 in renal failure correlated very well with renal function, it is likely that the expression of KSP32 reflects the integrity of a certain subset of important renal proximal and/or distal tubular cells that are susceptible to ischemic renal injury. Inspection of the kidney sections 24 and 96 h after injury indicated severe damage to the tubules, as evidenced by the low cellularity and void spaces. Cell necrosis and apoptosis, especially tubular cells, during ischemic ARF has been well documented (13, 22, 30, 41). It is during this period that KSP32 expression was completely lost (Figs. 4 and 5). Thus it is possible that KSP32 expression reflects the integrity of the renal tubules and damage to the tubular cells caused the downregulation or loss of KSP32.

Despite an extensive search, KSP32 was not found in several commonly used renal epithelial cell lines as well as cultured primary renal proximal tubule and cortical epithelial cells. The lack of KSP32 expression in cultured kidney cells was a surprise and a hindrance to studies of the physiological role of this protein. These observations may highlight the possibility that certain in vivo factors or cell-cell interactions may be required for maintaining KSP32 expression. Preliminary results by using kidney organ cultures also confirm that KSP32 expression is rapidly (<24 h) lost once the kidney is dissected. These in vitro results are reminiscent of the downregulation of KSP32 observed in the in vivo rat ischemic kidney models.

In summary, we have characterized a novel renal-specific gene termed KSP32. KSP32 shares little homology with other proteins in GenBank and its localization in renal medulla suggests a role in proximal tubular function. Expression of KSP32 is highly regulated in a rat model of ischemic ARF. Dramatic down-regulation is observed with 24 h of ischemic shock and the recovery of the KSP32 expression correlates with the recovery of renal functions.

We wish to thank Drs. Nicholas Laping, Laura Fitzgerald, and Richard Edward of the Department of Renal Pharmacology for helpful discussions.

REFERENCES