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Extracellular-regulated-kinase 5-mediated renal protection against ischemia-reperfusion injury

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Abstract

ERK5, a member of the mitogen activated protein kinase, expressed in the kidneys was smaller (~80 kDa) in apparent molecular mass compared to other organs (~120 kDa). A blocking peptide experiment confirmed that the ~80 kDa detected on Western blots was a specific band detected by the anti-ERK5 antibody. Expression of the known ERK5 variants ERK5a, b, c, and T confirmed that none of the known splice variants encoded for the renal-specific ~80 kDa protein. However, RT-PCR with primers targeting the potential splice sites did not reveal a novel transcript in the kidney. The smaller molecular mass of the kidney-specific ERK5-immunoreactive protein suggested that this cyto-protective molecule may not be fully functional in the kidneys. Lentivirus-mediated in vivo overexpression of full length ERK5 in the mouse kidneys provided protection against renal IR injury. The identity of the renal-specific ~80 kDa ERK5 remains unknown but a better understanding of the ERK5 expression and post-translational processing in the kidneys may reveal a novel strategy for renal protection.

Keywords

MAPK; ERK5; Western blot; mobility shift; viral transduction; IR injury

Introduction

Extracellular-regulated kinase (ERK)5 is an unique member of the mitogen activated protein kinase (MAPK) family with both kinase and transactivation properties (reviewed in [1]). Like other MAPKs, ERK5 transduces extracellular signal to intracellular events activated by growth factors, cytokines, and cellular stresses. Knock out mice work have documented the critical role of ERK5 in cardiovascular development since a global ablation of ERK5 or its upstream MEK5 and MEKK3 have resulted in embryonic lethality due to maldevelopment of the heart, poor angiogenesis, and endothelial apoptosis (reviewed in [2]). ERK5 also plays a role in post-natal physiology in mediating shear-flow-induced signaling in the vascular endothelium, regulation of cardiac ischemia-reperfusion (IR) injury, and

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transducing hyperglycemia-induced proapoptotic milieu in the streptozocin-induced diabetic mice model [3–6].

The ERK5 cDNA was originally found through a PCR screening of a human placental library using degenerate oligonucleotide primers targeting the highly conserved kinase domain of ERK1/2 [7] and simultaneously by another group as a binding partner to the MEK5 upstream kinase [8]. ERK5 proteins have a TEY-motif phosphorylated when activated identical to ERK1/2 and MEK5 is the only MAPK-kinase immediately upstream of ERK5 [9]. The ERK5 mRNA was found most strongly in the heart and lungs but also in many organs including the kidneys [7]. However, very little is known about the physiological role of ERK5 in non-cardiac or non-endothelial organs.

Phosphorylated ERK5 is found in the renal glomeruli in a rat strain genetically prone to type 2 diabetes mellitus. In vitro experiments with cultured renal mesangial cells confirmed that high glucose stimulation increased pERK5 and cell proliferation where both were inhibited by a pharmacological inhibitor of MEK5 indicating a correlation between mesangial cell proliferation and ERK5 activation [10]. Similarly, pERK5 expression increased in the renal mesangial cells in rats subjected to an experimental glomerulonephritis model. Short-inhibitory RNA inhibition of ERK5 in cultured renal mesangial cells demonstrated decreased viability to H_2O_2 or Ang II stimulation indicating an enhanced cell viability and possibly contributing to the accumulation of extracellular matrix and the pathogenesis of glomerulonephritis [11]. A recent study of the human kidneys confirmed the expression of ERK5 in the renal glomerular mesangium and in vitro studies with an over-expression of the dominant-negative truncated ERK5 supported the role of ERK5 in human mesangial cell proliferation, epidermal growth factor-induced cell contraction, and transforming growth factor (TGF)-β1-induced collagen I expression [12].

To gain a better understanding of the role of ERK5 in the kidneys, we examined the expression off ERK5 protein and studied the potential short-term protective effect of this MAPK in a murine kidney IR injury model.

Materials & Methods

Molecular constructs and expression in HEK 293 cells

The cDNA for N-terminal Xpress-tagged full-length mouse ERK5a (accession number NM_011841) subcloned in pcDNA3.1 was a gift from Dr. Junichi Abe (University of Rochester). mERK5b was created by replacing the wild-type codons encoding amino acids 70 – 77 with those encoding the residues MCGLLSRG by PCR. cDNA encoding the mERK5c was created by deleting the wild-type cDNA upstream of methionine 140 such that translation was only initiated from this start methionine. mERK5T cDNA encoding wild type aa 1–492 followed by LRGGVWAWWLSG unique to this variant was introduced by PCR. All PCR-derived constructs were sequenced to confirm the absence of unintended mutations.

For expression in human embryonic kidney (HEK) 293 cells (CRL-1573, ATCC), cDNAs were subcloned into the CMV promoter-driven eukaryotic expression vector pCI/neo (Promega). Approximately 5×10^5 cells cultured in Dulbecco's modified Eagle's medium (4.5 g/ L glucose) and supplemented with 10% fetal bovine serum, 100 U/ mL penicillin and 100 μg/ mL streptomycin were plated onto each gelatin-coated well of a 6-well tissue culture plate and 2 μg plasmid DNA was transfected using Lipofectamine 2000 (Invitrogen) following the manufacture's recommended protocol. Cells were harvested 48 hrs after transfection and processed for Western blot experiments.

RT-PCR

Total RNA was extracted from 30 mg minced freshly harvested organs according to the manufacturer's protocol (RNeasy, Qiagen). Reverse transcription (RT) and PCR was performed using the SuperScript II kit (Invitrogen) with 300 ng total RNA and the primer pairs indicated. RT at 50 C^o×30 min was followed by 40 cycles of PCR amplification (94 C^o×30 s, 64 C^o×30 s, 72 C^o×60 s). An alternative 2-step Rt-PCR where RNA was first reverse transcribed to cDNA using a non-specific proprietary primer mixture (QuantiTect Reverse Transcription Kit, Qiagen) followed by PCR amplification for the selective products gave identical results.

Lentivirus

Lentivirus vectors were created by a triple transfection of HEK293T cells (CRL-11268, ATCC) with a shuttle vector with the mERK5a subcloned, pVSVG for psuedo-typing, and Δ8.9 packaging-plasmid. For expression of gene-of-interest, an in house-modified pLL 3.7 shuttle vector (VRMC-39, ATCC) where the mouse CMV promoter drives both the expression of the gene and the EGFP reporter was used. Culture media containing virus particles were collected 48 and 72 h after transfection and concentrated by ultra-centrifugation [13] and the viral titer was estimated by transducing and counting fluorescence positive cells. The final titer obtained was $2.5 – 5.8\times10^3$ t.u./μl starting from four 15 cm plates of HEK293T cells.

Animals

The functional consequence of overexpression of ERK5 was examined by an intra-renal parenchyma injection of mERK5a-expressing lentivirus according to a procedure originally described by Gusella et. al. [14] and previously implemented by our group [15]. In mice anesthetized with pentobarbital (~50 mg/ kg), the left renal pedicle was occluded and a 31G needle inserted at the lower pole of the kidney was pushed toward the upper pole. As the needle was slowly removed 100 μl of in vivo grade lentivirus was injected and the pedicle occlusion removed. The entire procedure took less than 15 s. Three days after the viral transduction, the mice were anesthetized and placed supine on a heating pad under a warming light to maintain the body temperature at 37 C^o. A midline incision was made and a right nephrectomy performed. A micro aneurysm clip was used to occlude the left renal pedicle for 30 minutes as this duration of ischemia resulted in reproducible IR injury without significant mortality. Upon removal of the clip, 0.5 ml warm saline was given intra-peritoneally and the wound closed in two layers. This IR injury protocol was approved by the IUCAC of Columbia University. The renal function was assessed by measurement of plasma creatinine 24 h after renal ischemia by a colorimetric method based on the Jaffe reaction [16]. In some animals, the kidneys were excised after perfusion with saline to rid of blood, immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), then soaked in phosphate-buffered 30% sucrose overnight, optimal cutting temperature compound mounted, frozen tissue sections (15 μm) cut on a cryostat, and thaw-mounted on glass slides. The sections were stained with hematoxylin and eosin (H&E), and photographed on an Olympus IX50 microscope equipped with a Cooke Sensicam camera (Cooke Corp).

Western blot

Protein lysate prepared from fresh organs isolated from adult mice (adult C57BL ~ 25 gm) were studied using Western blot. Mice under deep pentobarbital anesthesia were trans-cardially perfused with 100 ml of saline to rid of blood. The harvested organs were minced, dissolved in RIPA buffer (1% Nonidet P-40, 10 mM Tris pH7.5, 50 mM NaCl, 30 mM NaPPi, 50 mM NaF, 1% Triton X 100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl-

sulfate, 1 mM phenylmethylsulphonyl fluoride, 0.1mM iodoacetamide, 1mM Na_3VO_4, 50uM N-ethyl maleamide) supplemented with a protease inhibitor cocktail, and further dissociated by grinding in an eppendorf tube on ice with a pestle. The undissolved tissue particles were clarified by centrifugation and the total protein concentrations were measured by BCA protein assay kit (Pierce). Proteins were denatured and reduced by boiling in a SDS and β-mercaptoethanol containing buffer prior to loading the gel. Electrophoresis was performed on 10% polyacrylamide gel. Proteins were transferred from the gel onto nitrocellulose membrane (BioRad). The blots were blocked for 1 h with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature and incubated with primary antibodies in TBST containing 3% skim milk overnight at 4°C or for 2 h at room temperature. The ERK5 protein expression was probed using two different commercially available anti-ERK5 antibodies (1:2000, #3372, Cell Signaling Technology or 1:2000, #07–039, Millipore) and the equal protein loading confirmed by probing for GAPDH (1:100000, #RGM2, Advanced Immuno Chemical Inc). Preliminary experiments confirmed identical results between the two anti-ERK5 antibody and the Cell Signaling Technology antibody where the blocking antibody was commercially available was used for the subsequent experiments. The ERK5 blocking peptide (#1617B, Cell Signaling Technology) was used at the final concentration of 1 μg/ ml at the time of primary antibody exposure. The blots were washed three times and incubated with HRP-conjugated secondary antibody in TBST containing 1% skim milk at room temperature for 1 h. After rinsing with TBST, proteins were detected by enhanced chemiluminescence method (Western Lightning Plus, Perkin Elmer).

Results

Immunoblotting of protein extracts from freshly isolated mouse organs with anti-ERK5 antibody revealed the expected ~120 kDa band [8, 17] in all organs examined except for little expression in the skeletal muscle and a band ~80 kDa in the kidney (Figure 1A). Since the detected band in the kidney was not the expected molecular mass, we confirmed the specificity of the anti-ERK5 antibody with a blocking peptide. The blocking peptide blocked the immunoreactivity at ~120 kDa present in HEK293 cells transfected with ERK5a cDNA and brain extracts (lanes 1 and 2) and the ~80 kDa band in the kidney (lane 3) while leaving the non-specific band around 75 kDa in the brain sample intact (Figure 1B).

What could be the identity of the ~80kDa anti-ERK5 antibody immunoreactive band specifically observed in the kidney lysate? We began by expressing the known ERK5 splice variants reported in the literature. In the mouse, 3 splice variants resulting from different selection of donor and acceptor splice sites in the 1st and/or 2nd introns gives mERK5a (806 aa, 88kDa), mERK5b (737 aa, 80 kDa), and mERK5c (667 aa, 72 kDa) have been reported [18]. Another truncation variant resulting from an alternate splicing of the 4th intron introducing an in-frame premature stop codon gives mERK5-T (506 aa, 56 kDa) [19] (Figure 2A). We expressed these four known mERK5 splice variants in HEK293 cells and performed a Western blot running the samples from heart and kidney simultaneously. All expressed mERK5 cDNA ran on the SDS gel with a greater molecular mass than expected. The anti-ERK5 immunoreactivity of lysate from the heart ran identical to mERK5a while none of the expressed clones ran at the same apparent molecular mass with the sample form the kidney.

We sought for potential kidney-specific ERK5 mRNA variant to explain the ~80kDa kidney-specific immunoreactive band. The coding sequence of ERK5 consists of exons 2–7 with the intervening introns (Figure 3A). We designed primers spanning the introns and probed for novel mRNAs by rt-PCR of RNA isolated from a freshly harvested mouse heart

and kidney. Rt-PCR amplified bands of the expected sizes but no new band that may correspond to a novel kidney-specific mRNA transcript was found (Figure 3B).

The identity of the ~80 kDa anti-ERK5 antibody immuno-reactive band only present in the kidney is unknown but we suspected that the renal ERK5 may be deficient in function compared to the larger mERK5a as has been shown for the shorter mERK5b, c and T variants. Since mERK5a has been demonstrated to protect various organs from cellular stress leading to apoptosis, we explored for a potential functional significance of overexpressing the full length mERK5a in protecting the kidney against IR injury.

Lentivirus expressing the full length mERK5a was created by a triple transfection of the shuttle vector, VSVG psuedo-type, and the packaging vector Δ8.9. The shuttle vector employed was an in-house-modified pLL3.7 that allowed simultaneous expression of the mERK5a and EGFP reporter downstream of an IRES sequence [20]. Ultra centrifugation-concentrated in vivo-grade lenti-mERK5a-EGFP(~5×10^5 t.u.) was injected into the left kidney of mice, allowed to survive for 3 days and subjected to an IR injury model with right nephrectomy and left complete ischemia for 30 minutes. Serum creatinine 24 hours after IR injury was significantly less in the mERK5a-overexpressing group suggesting a protective effect of the full length mERK5a against renal IR injury (Fig 4A). Histological evaluation of the kidneys was consistent with the protective effect of the mERK5a-expressing kidney will better preservation of the cellular architecture compared to the control mice not injected with the lenti-mERK5a-EGFP subjected to IR injury (Fig 4B). Proper transduction of the kidney after lenti-mERK5a-EGFP injection was confirmed by a rt-PCR (Fig 4C) and the presence of the full-length mERK5a immunoreactive band of approximately 120 kDa was documented by a Western blot (Fig 4D).

Discussion

An anti-ERK5 antibody immuno-reactive protein of apparent molecular mass of ~80 kDa was expressed in the kidney smaller in apparent molecular mass from those expressed in other organs examined. We screened for a potential new kidney-specific ERK5 transcript but found none; we do not know why the apparent protein mass was smaller in the kidney but suspected that the small renal-specific ERK5 was not fully functional. Lentivirus-mediated overexpression of full length mERK5a cDNA resulted in expression of ERK5 immunoreactive protein of ~120 kDa and provided protection against in vivo renal IR injury. Understanding the unique processing and/or expression of this MAPK in the kidneys may allow exploitation of the ERK5 signaling pathway for renal protection in contrast to the hitherto suggested pathological role of long-term ERK5 activation in several renal diseases.

Anomalous apparent molecular mass on SDS-PAGE can result from unaccounted post-translational processing of the protein, covalent conjugation with another partner protein, or simply due to some intrinsic property of the protein (very basic or acidic proteins, various glycoproteins or lipoproteins) such that SDS is unable to properly surround the protein allowing it to move along the electric field according to its molecular mass [21]. ERK5 is a complex protein with several well described post-translational modifications including phosphorylation and small-ubiquitin-like modifier (SUMO)ylation [5, 17]. However, even the larger SUMOylation of a target protein should only results in an expected mobility shift of ~10 kDa [22] and insufficient to account for the 40kDa mobility shift observed for ERK5 unless multiple sites are SUMOylated. Nevertheless, we probed the ERK5 membrane with anti-SUMO1/2 antibody but found no immunoreactivity of the ~120 kDa band (data not shown) consistent with our previous observation that SUMOylated ERK5 migrates at an apparent molecular mass greater than ~120 kDa on a denaturing SDS-PAGE [5]. We do not know why the ERK5 protein shows the mobility shift but suspect it is simply due to some

intrinsic property of this protein. For the transcription factor Pdx-1, a similar mobility shift from the expected mass of 31 kDa to the apparent mass of 45kDa was determined to be most likely due to the large number of prolines (around 16%) present in that protein [23]. The full-length mERK5a has 100 prolines or 12.4% of the total 806 residues with a calculated isoelectric point of pH 5.43.

Several reports have appeared in the literature confirming expression of ERK5 in the kidney and its potential role in renal diseases mostly using immunohistochemical techniques [10–12, 24, 25]. Only one study to date has reported the actual molecular mass of the immunoreactive band. Dorado et. al. [12] showed a ~120 kDa ERK5 band on a Western blot of human kidney specimen processed within 3 hrs of surgical excision. We do not believe our observation of a ~80 kDa band in the mouse kidney is species specific but rather due to the fact that mice samples were processed immediately after harvest and suggest that the intrinsic ERK5 expressed in the kidney in situ is the ~80 kDa species. Cultured cell lines of renal origin (e.g. HEK 293 or HK1cells) or acutely isolated and cultured primary renal tubular cells demonstrated ERK5 immuno-reactive band at 120 kDa (data not shown).

ERK5 in the kidney has been reported to be expressed mainly in mesangial cells [10–12] although the proximal tubular cells also express this MAPK [24]. Pharmacological and molecular biological inhibition of ERK5 or conversely ligand-activation of the ERK5 pathway suggested a proliferative role of ERK5 activation on cells. Our results with the lentivirus-mediated over-expression of ERK5 in the kidney may additionally serve a protective role against IR injury. In this regards, ERK5 shares with the closely related ERK1/2 MAPK that prolonged activation may lead to renal diseases [26] while brief activation may be protective consistent with the cell proliferative actions of ERK5 (reviewed in [1]). Pretreatment of the kidneys with ligands such as TGF-β demonstrated to activate ERK5 [25] could be a novel method to protect the kidneys although whether activation of the endogenous smaller molecular mass ERK5 will be protective is not known.

Highlight

- ERK5 in the kidney has a smaller apparent molecular mass.
- This is distinct from all known ERK5 variants.
- Rt-PCR failed to detect novel ERK5 transcripts in the kidney.
- Lentivirus-mediated expression of full-length ERK5 protects against IR injury

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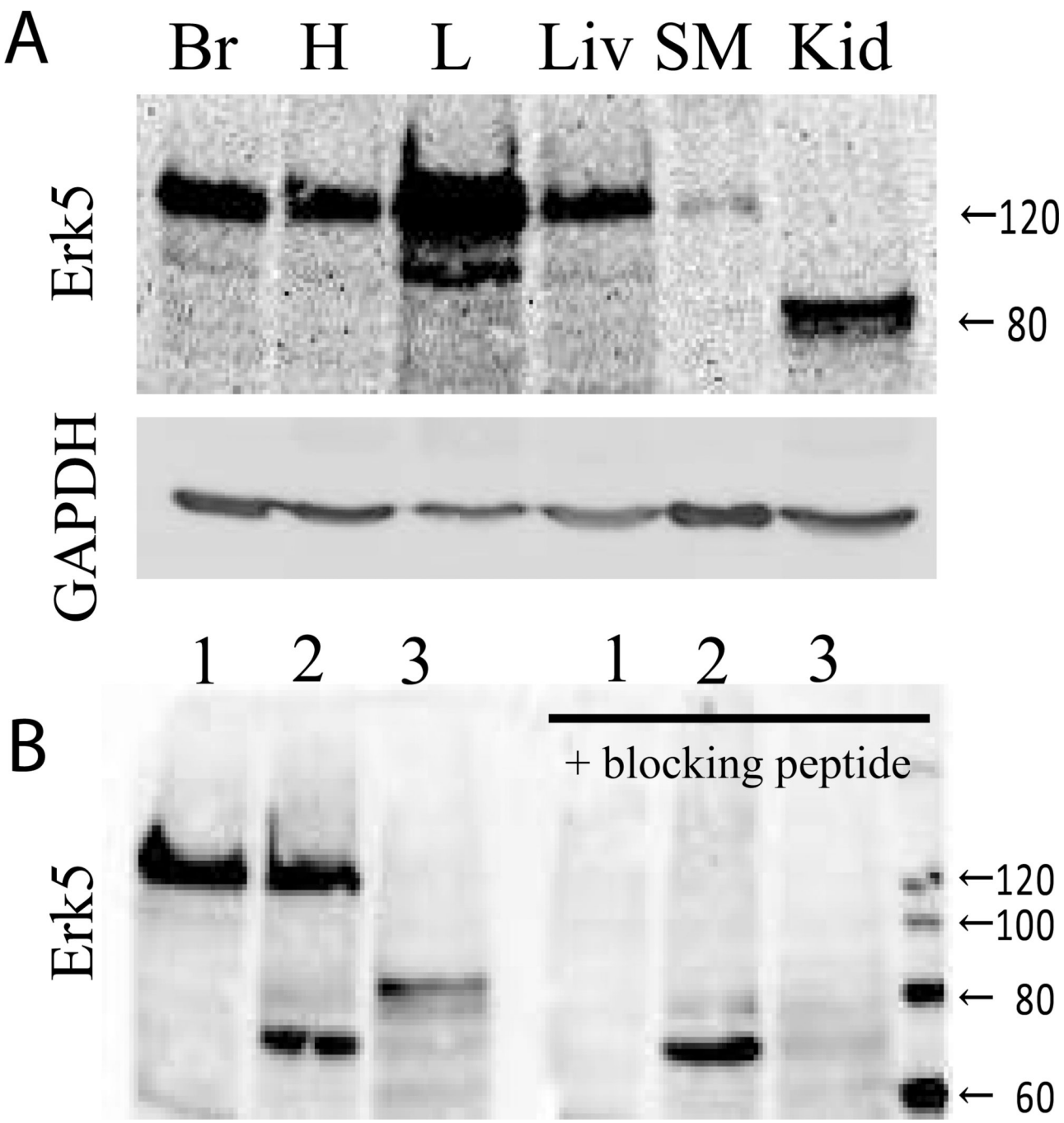

Figure 1. Renal specific expression of a novel ERK5 antibody immunoreactive protein

A Protein lysates (30 μg) from different organs were separated by SDS-PAGE and probed with the anti-ERK5 antibody. Most organs showed a ~120 kD band except the kidney that showed a smaller ~80 kD band. Br: Brain, H: heart, L: lung, Liv: liver, SM: skeletal muscle, Kid: kidney. The lower panel is a reblot of the same membrane with an anti-GAPDH antibody. **B**. Both the ~120 and ~80 kD immunoreactive bands were abolished by pre-incubation (bar) with the blocking peptide prior to the application of the anti-ERK5 antibody while the non-specific immunoreactive bands including the prominent 75 kD band seen only in the brain lysate remained. Lysates from: 1. HEK293 cells transfected with ERK5a cDNA, 2. brain, and 3. kidney. Approximate molecular mass are denoted to the right. The blot is

representative of at least 4 blots with protein extracted from organs harvested from different mice.

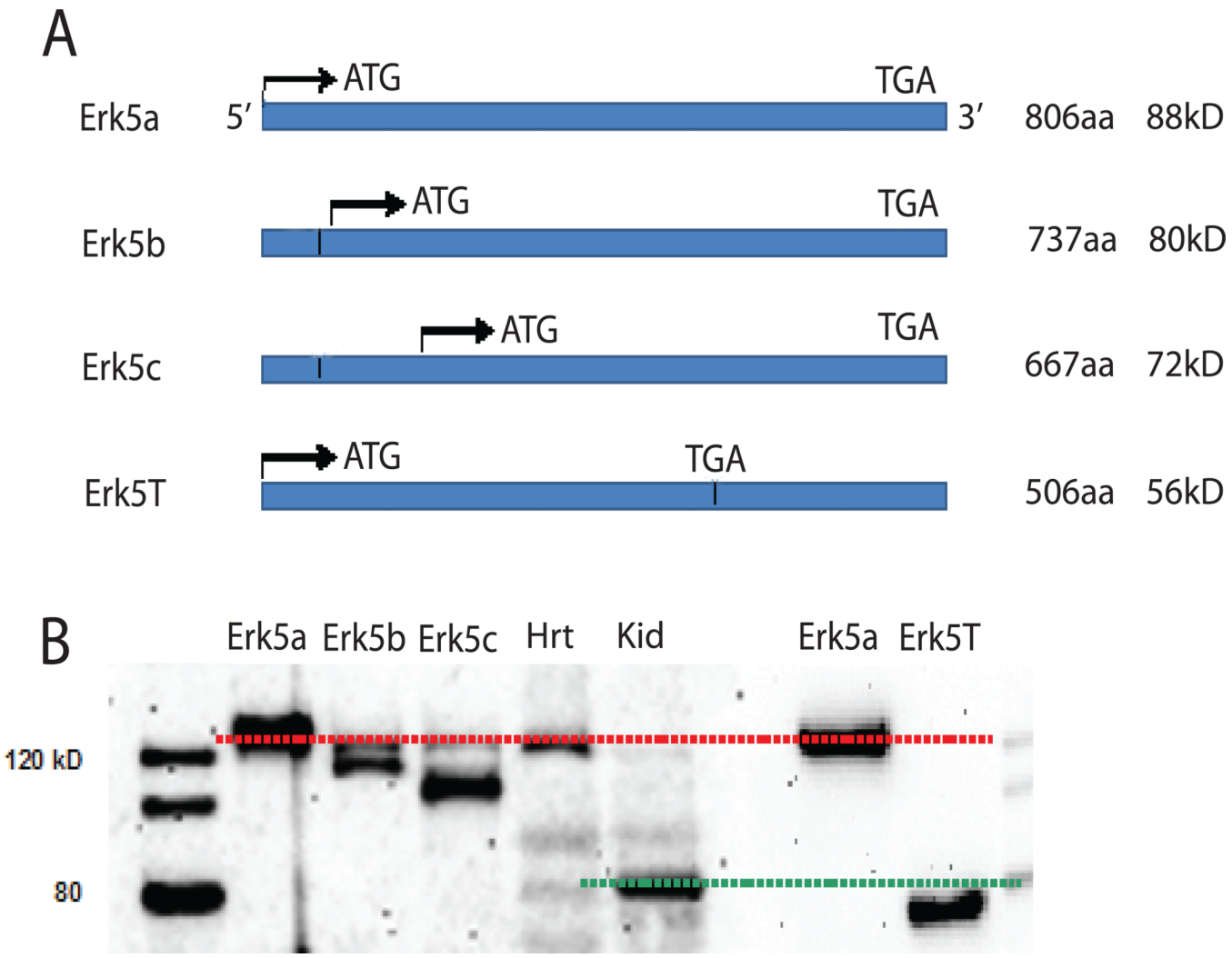

Figure 2. Expression of the known ERK5 isoforms did not result in the renal-specific ~80 kD protein band

A. A schematic of the known mERK5 variants denoting the start and the stop codons. The number of encoded amino acids and the expected molecular mass are shown to the right. **B**. A representative Western blot of lysates obtained from HEK293 cells transfected with the denoted ERK5 cDNA (20 μg protein loaded) or from the organs (50 μg protein loaded). HEK293 cells express endogenous ERK5-IR band at ~120 kD faintly visible in the ERK5b and c lanes.

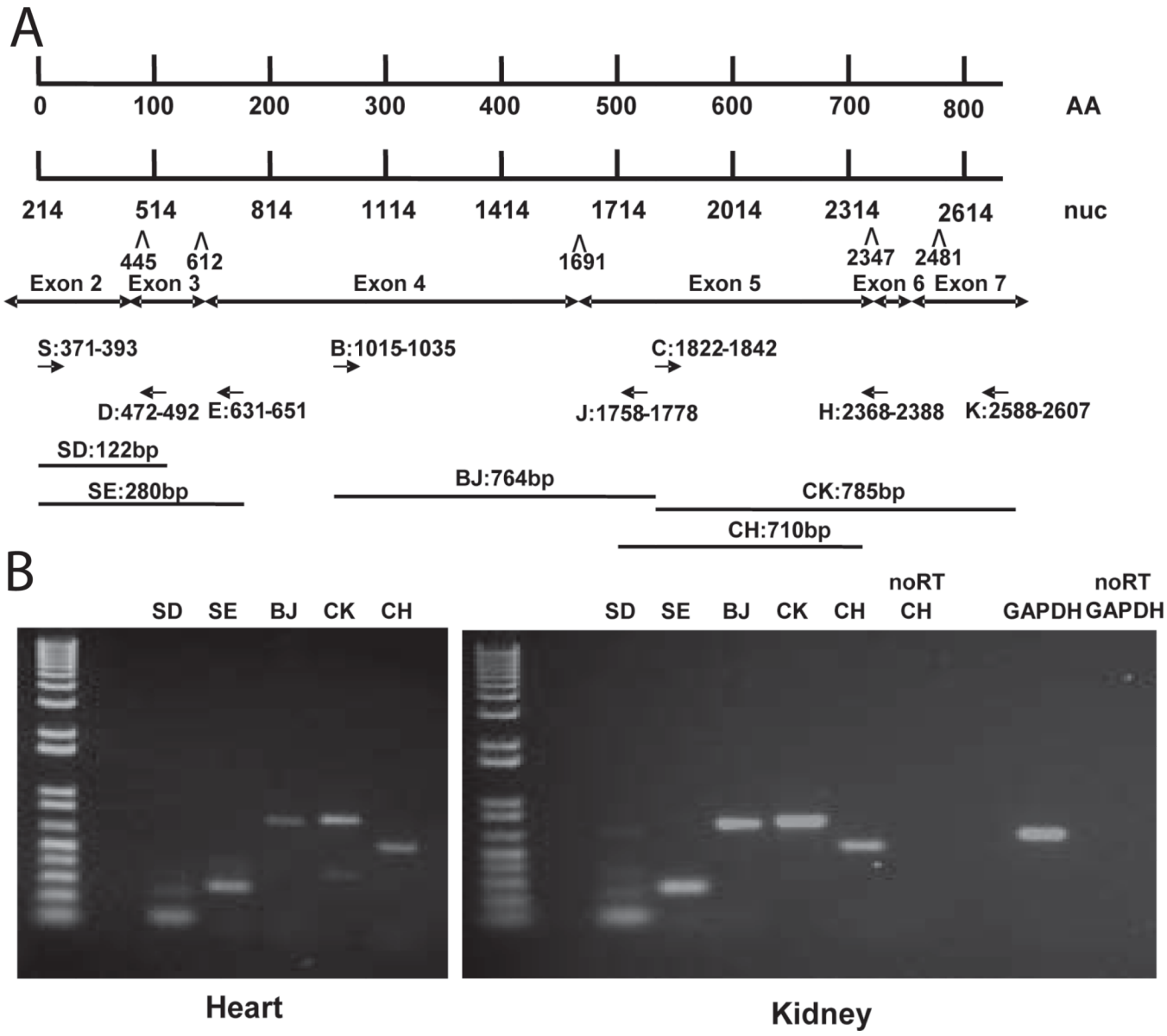

Figure 3. RT-PCR across the exon-intron boundaries of total RNA harvested from the organs did not yield new ERK5 splice variants unique to the kidney

A. A schematic diagram denoting the amino acid, nucleotide numbers, genomic structure of the mERK5 gene, and the size of the introns. The numbering is based on the full length mERK5a cDNA (accession number NM_011841). Locations of the primers used for the PCR amplification are noted with the corresponding nucleotide numbers. The expected PCR product size for the mERK5a cDNA is noted. The mouse GAPDH primers used as control were Forward: ttcaccaccatggagaaggcc and Reverse: ccctgttgctgtagccgtatt with an expected product size of 650 bp. **B**. Agarose gel electrophoresis of the RT-PCR products using mRNA isolated from the heart (left) or the kidney (right). The expected products are denoted on top and no RT negative controls gave no products (CH and GAPDH).

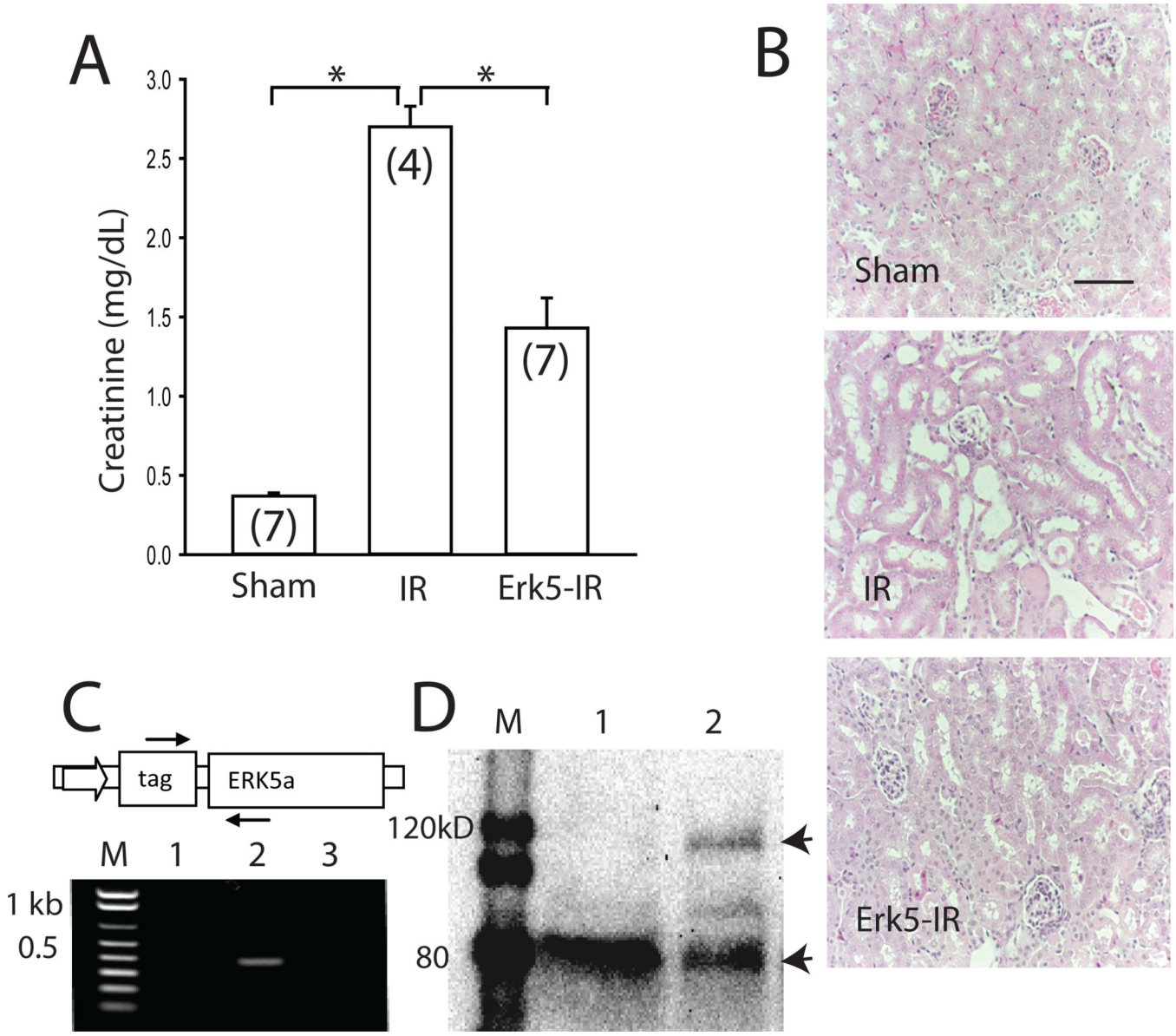

Figure 4. Lentivirus-mediated intra-renal overexpression of full-length ERK5a confers protection against IR injury

A. Serum creatinine (mg/ dL) of mice 24 hrs after a 30 min IR injury to the left kidney and right nephrectomy. The * denotes P<0.001 for a 2-sided Student t-test. Sham denotes mice subjected to laperotomy only without renal IR. The bars are mean±S.E.M. and the number of mice denoted in parentheses. **B**. Photomicrographs of H&E stained renal cortical sections from Sham (non-injected and not subjected to IR injury), IR (non-injected and subjected to IR injury), and ERK5-IR (lenti-ERK5-EGFP injected and subjected to IR). Bar=50 μm. **C**. An agarose gel picture of RT-PCR products of kidney total RNA harvested from non-injected (1), lenti-ERK5-EGFP injected (2), and same but without reverse transcription (3). The PCR primers spanned the 5'Xpress-tag (cgggatctgtacgacgatgacgataag) and ERK5a coding sequence (caccacatcaaaagcattagg) with an expected product size of 380 bp and only amplifying the virally-transduced transgene. M=nucleotide base pair marker. **D**. Western blot probed with anti-ERK5 antibody of kidney lysates prepared from non-injected (1) or lenti-ERK5-EGFP injected organs harvested 3 days after the injection. Arrows denote anti-

ERK5 immuno-reactive bands but the upper ~120 kD band was seen only in the injected kidney and not in the control uninjected kidney. M=molecular mass marker.