HETEROGENOUS NUCLEAR RIBONUCLEOPROTEIN K MODULATES ANGIOTENSINOGEN GENE EXPRESSION IN KIDNEY CELLS

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Running Title: Heterogenous nuclear ribonucleoprotein K and angiotensinogen

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Summary

The present studies aimed to identify the 70-kiloDalton nuclear protein that binds to an insulin-responsive element in the rat angiotensinogen gene promoter and to define its action on angiotensinogen gene expression. Nuclear proteins were isolated from rat kidney proximal tubular cells and subjected to 2-dimensional electrophoresis. The 70-kiloDalton nuclear protein was detected by Southwestern blotting and subsequently identified by mass spectrometry, which revealed that it was identical to 65-kDa heterogenous nuclear ribonucleoprotein K (hnRNP K). HnRNP K bound to insulin-responsive element of rat angiotensinogen gene was revealed by gel mobility shift assay and chromatin immunoprecipitation assay. HnRNP K inhibited angiotensinogen mRNA expression and promoter activity. In contrast, hnRNP K down-expression by small interference RNA enhanced angiotensinogen mRNA expression. Moreover, hnRNP K interacted with hnRNP F in pull-down and co-immunoprecipitation assays. Co-transfection of hnRNP K and hnRNP F further suppressed angiotensinogen mRNA expression. Finally, \textit{in vitro} and \textit{in vivo} studies demonstrated that high glucose increases and insulin inhibits hnRNP K expression in rat kidney proximal tubular cells. In conclusion, our experiments revealed that hnRNP K is a nuclear protein that binds to the insulin-responsive element of the rat angiotensinogen gene promoter and modulates angiotensinogen gene transcription in the kidney.

Introduction

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease (ESRD), accounting for 30-50\% of all new ESRD cases in North America (1-3). Both clinical and animal studies indicate that intensive insulin therapy and prolonged treatment with angiotensin-converting enzyme (ACE) inhibitors or angiotensin II (Ang II)-AT\(_1\) receptor blockers delay the progression of nephropathy in diabetes, but neither strategy cures nephropathy (4-12). While such results support the concept that hyperglycemia and the renin-angiotensin system (RAS) activation are involved in the development and progression of DN, the molecular mechanism(s) linking hyperglycemia to RAS activation remain largely undefined.

Angiotensinogen (AGT), a glycoprotein consisting of 452 amino acid residues with an apparent molecular weight (MW) of 62-65 kilodalton (kDa), is the sole substrate in the RAS cascade (13, 14). AGT is principally produced by the liver and cleaved by renin from the kidney to form angiotensin I (Ang I), which is then further processed by ACE to form Ang II. The existence of an intrarenal RAS is now generally accepted (15, 16). Renal proximal tubules (RPTs) contain all components of the RAS, including messenger RNAs and proteins, such as AGT, renin, ACEs, and Ang II receptors (17-24). Most recently, we reported that RAS blockade decreases blood pressure and proteinuria in transgenic mice overexpressing rat AGT (rAGT) gene in the kidney (25). These observations indicate that the local formation of Ang II may play an important role in diabetic nephropathy.
role in the development of nephropathy in diabetes.

Our laboratory has established that high glucose (i.e., 25 mM) stimulates rAGT gene expression in IRPTCs (26-29). RAS blockers and stable transfer of antisense rAGT cDNA into IRPTCs inhibit transforming growth factor-beta 1 (TGF-β1) gene expression and cellular hypertrophy in high glucose (30, 31). These investigations strongly indicate that rAGT and TGF-β1 gene expression are essential for the high-glucose effect on IRPTC hypertrophy and kidney injury. We have also established that insulin inhibits the stimulatory effect of high glucose levels on rAGT gene expression and the induction of hypertrophy in IRPTCs (32-43). Moreover, a putative insulin-responsive element (IRE) containing nucleotides N-878 to N-864 (5′ CCT TCC CGC CCT TCA 3′) upstream of the transcription start site of the rAGT gene promoter has been identified, and it binds to 2 major nuclear proteins with apparent MW of approximately 48 and 70 kDa from IRPTCs (34). We recently reported that the 48-kDa nuclear protein is identical to 46-kDa heterogeneous nuclear ribonucleoprotein F (hnRNP F) (35). Furthermore, transient transfer of sense and antisense hnRNP F cDNA respectively inhibits and enhances rAGT gene expression in IRPTCs (35).

The present studies aimed to identify the 70-kDa nuclear protein and to investigate its action on rAGT gene expression. We identified the 70-kDa nuclear protein as 65-kDa heterogeneous nuclear ribonucleoprotein K (hnRNP K) by 2-dimensional (2-D) electrophoresis and mass spectrometry (MS). Recombinant hnRNP K bound to rAGT-IRE, as shown by gel mobility shift assay (GMSA) and chromatin immunoprecipitation assays. Over-expression of hnRNP K attenuated rAGT mRNA expression and rAGT gene promoter activity in IRPTCs. In contrast, down-expression of hnRNP K by small interference RNA enhanced rAGT gene expression. Moreover, hnRNP K was pulled down and co-immunoprecipitated with hnRNP F. Cotransfection of hnRNP K and hnRNP F further suppressed rAGT mRNA expression. Finally, in vitro and in vivo studies revealed that high glucose or hyperglycemia increased and insulin inhibited hnRNP K expression in rat kidney proximal tubular cells. These experiments demonstrated that 65-kDa hnRNP K is a nuclear protein that binds to the rat ANG gene promoter and modulates AGT gene expression in the kidneys.

EXPERIMENTAL PROCEDURES

D(+)-glucose, D-mannitol and insulin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Insulin implant ((Linplant) and gamma-[32P-ATP] (3,000 Ci/mol) were obtained from Linshin Ltd. (Scarborough, ON, Canada) and Amersham-Pharmacia Biotech (Baie d’Urfé, QC, Canada), respectively. Plasmid containing full-length hnRNP K cDNA (pcDNA 3/Flag-hnRNP K) and rabbit polyclonal antiserum (#54) recognizing hnRNP K (QNSVKQYADVEGF corresponding to amino acids 452 to 464 of human hnRNP K) were generated (K.B.’s lab.) as described previously (36). Mouse monoclonal antibody against human hnRNP K/L (clone 3C2), a gift from Dr. Gideon Dreyfuss (Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA), has been reported elsewhere (37). Rabbit polyclonal anti-TATA box-binding protein (TBP) (sc-273) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The bacterial expression vector pGex 4T-3 and mammalian expression vectors, pcDNA 3.1 and pRC/RSV were purchased from Amersham-Pharmacia Biotech and InVitrogen, Inc., respectively. Restriction modified enzymes were acquired from either InVitrogen, Inc., Amersham-Pharmacia Biotech or La Roche Biochemicals (Laval, QC, Canada).

Oligonucleotides for rAGT-IRE N-882 to N-855 (5′ CCT CCC TTC CGC CCC TTC ACT TTC TAG T 3′) (34), mutants of rAGT N-882 to N-885 (M1, 5′ CCT CCC TTC ACT TAG T 3′; M2, 5′ CCT CCC TTA AAT AAG ACC ACT TTC TAG T 3′; M3, 5′ CCT CCC TTC CCT TCC TTC ACT TTC TAG T 3′; M4, 5′ CCT CCC TTC CCT CCC TTC ACT TTC TAG T 3′), concanemeric wide type (wt) rAGT-IRE motif (3 x N-878 to N-864, 5′ CCT CCC CGC CCT TCA CCT TCC CCG CCC CCT CTC 3′), concanemeric mutant rAGT-IRE motif (3 x N-878 to N-864, 5′ CCT CCC TCA CCT TCC CCT CCG CCC CCT CTC 3′), IRE of human glyceraldehyde phosphate dehydrogenase gene (hGAPDH-IRE, N-473 to N-477, 5′ CCA ACT TTC CGG CCT CTC 3′),
AGC CTT TGA A 3’) (38), IRE of rat glucagon gene (N-267 to N-242, 5’ AGT TTT CAC GCC TGA CTG AGA TTG A 3’) (39), and the consensus Sp1-binding site (5’-TCG CCC CGC CCC CGA TCG AAT-3’) (40) were synthesized by InVitrogen, Inc. as reported previously (35).

The plasmid containing the concatemeric wide type and mutant rAGT-IRE motif DNAs were constructed by inserting the double-stranded concatemeric wide type or mutant rAGT-IRE motif oligonucleotide with the Not-1 enzyme restriction site added on both termini into the plyconal site of pcDNA 3.1 by conventional methodology. The double-stranded concatemeric wide type or mutant rAGT-IRE motif DNA fragment was then excised from the plasmid and treated with alkaline phosphatase and used for labeling as probe.

**Cellular Nuclear Extract Preparation**

IRPTCs from passages 12 to 18 were utilized. The characteristics of IRPTCs, which express the mRNA and protein of rAGT, renin, ACE, and Ang II receptors, have been described previously (41). IRPTC nuclear extracts were prepared from 20 plates (150 x 20 mm), each containing confluent IRPTCs previously incubated in Dulbecco’s modified Eagle medium (DMEM) with 5 mM glucose plus 20 mM D-mannitol, 25 mM glucose, or 25 mM glucose plus insulin (10^{-7} M) for 24 h according to the method of Henninghausen and Lubon (42) with slight modifications (34, 35).

**2-D Electrophoresis**

2-D electrophoresis was carried out with the IPGphor Isoelectric Focusing Unit (Amersham-Pharmacia Biotech) as previously described (35). For 2-D separation, the IPG strips were placed above 10% polyacrylamide gel containing SDS and electrophoresed (SDS-PAGE) (35). Amersham’s rainbow markers served as MW markers. IRPTC nuclear extracts (100 µg) were run on the same 10% SDS-PAGE as the controls. Each sample was divided into 2 strips for 2-D electrophoresis. One gel was stained with Coomassie Brilliant Blue R-250 (Amresco Inc., Solon, OH, USA) to visualize proteins. The other was electrotransferred to a Hybond C-extra membrane (Amersham-Pharmacia Biotech) for Southwestern blotting.

**Southwestern Blotting**

Southwestern blotting was performed according to the procedure of Kwast-Welfeld et al. (43) with slight modifications (34, 35). Briefly, IRPTC nuclear proteins (200 µg) were resolved on a 4 to 20% SDS-PAGE gradient or on 10% SDS-PAGE (44), then electrotransferred to a Hybond C-extra membrane. The membrane was incubated with 10% (W/V) non-fat milk proteins and then washed at least twice with binding buffer containing 0.25% non-fat milk proteins. Subsequently, it was hybridized overnight with 32P-labelled rAGT-IRE DNA (approximately 1.0 to 2.0 pmol; 10^6 cpm/ml) in binding buffer containing 0.25% non-fat milk proteins and 300 µg/ml non-denatured herring sperm DNA at 4°C. The membrane was finally washed, air-dried and exposed for autoradiography.

**Matrix-assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS)**

Spots on the gel corresponding to positive signals of the Southwestern blot membrane were picked up for MALDI-MS. All MALDI-MS analyses were performed at the Quebec Genome Centre (McGill University, Montreal, QC, Canada). Briefly, protein samples were first cleaved by trypsin and then subjected to MALDI-MS. MALDI-MS analysis was conducted at 20-kV accelerating voltage and 23-kV reflecting voltage. For protein identification, peptide mass fingerprints were searched by the Mascot program developed by Matrix Science Ltd. (freely accessible on http:\www.matrixscience.com).

**Expression of Recombinant hnRNP K**

Murine hnRNP K cDNA (36), with the Not 1 enzyme restriction site added on the 5’ and 3’ ends of sense and antisense primers, respectively, was subcloned at the polycional site (Not 1) of the bacterial expression vector pGex 4T-3 by conventional methodology. E. Coli BL-21 cells (Amersham-Pharmacia Biotech) were transformed by pGex 4T-3 containing hnRNP K cDNA. Expression of the fusion protein [GST fused with hnRNP K (GST-hnRNP K)] in BL-21 cells was induced by the addition of 1 mM isopropylthiogalactoside (IPTG) in the culture medium with incubation for 4 h at 37°C. The bacteria were then harvested, and GST-hnRNP K
fusion proteins were purified from the bacterial extracts by GST affinity column chromatography according to the manufacturer’s protocol (Amersham-Pharmacia Biotech). The purified GST-hnRNP K fusion proteins were tested in GMSAs.

**GMSAs**

These assays were performed according to the methodology described elsewhere (34, 35), employing labeled rAGT-IRE DNA as probe. Briefly, the rAGT-IRE DNA fragment was 5’ end labeled with [\(\gamma^{32}\)P]-ATP by T4 polynucleotide kinase. Purified GST-hnRNP K fusion proteins (1.5 \(\mu\)g) or GST (5 \(\mu\)g) in the presence of 1 \(\mu\)g of poly(dI/dC) in 20 mM Heps (pH 7.6), 1 mM EDTA, 50 mM KCl, 2 mM spermidine, 1 mM DTT, 0.5 mM PMSF and 10% glycerol (V/V) were incubated for 30 min on ice. Subsequently, the 5’-labelled probe (~0.1 pmol) was added and further incubated for 45 min on ice. The mixture was run on 6% (W/V) non-denaturing PAGE and exposed for autoradiography.

In competition assays, 100-fold molar excess of unlabeled DNA fragments was added to the reaction mixture and incubated for 30 min on ice before incubation with the labeled probe.

**Chromatin immunoprecipitation (ChIP)**

ChIP analysis was performed according to the methodology of Kuo and Allis (45) with slight modifications (46). Briefly, 0.4 ml of 37% formaldehyde was added to 10 ml of overlaying media of IRPTC culture for 15 min at 4°C. After cross-linking, the cells were harvested, washed twice with 1 ml of PBS in Eppendorf tubes, and then lysed with 0.5 ml of immunoprecipitation (IP) buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% NP-40, 50 mM Tris-HCl, pH 7.5, 0.5 mM DTT) containing the following inhibitors: 10 \(\mu\)g/ml leupeptin, 0.5 mM PMSF, 30 mM p-nitrophenol phosphate, 10 mM NaF, 0.1 mM Na3VO4 and 10 mM \(\beta\)-glycerophosphate. After 1 wash with IP buffer, the pellet was suspended in 1 ml IP buffer and sheared in a Branson sonicator with 10-s cycles, 1 pulsed and 1 continuous, for 10 min at an output 3 and 80% duty cycle. Pull-downs were done using anti-K protein antibody with or without blocking peptide (100 \(\mu\)M) and protein A beads (Amersham-Pharmacia Biotech). The beads were washed 5 times with 1 ml of IP buffer without inhibitors. DNA was eluted twice from the beads with 250 \(\mu\)l of elution buffer (1% SDS, 0.1 M NaHCO3) for 15 min with periodic vortexing (at room temperature). Cross-linking was reversed by adding 1 \(\mu\)l of 10 mg/ml RNase and 5 M NaCl to a final concentration of 0.3 M and incubating the tubes at 65 °C for 4 hours. After adding 2.5 volumes of 100% ethanol, the pellet was precipitated overnight at -20°C. DNA was pelleted and resuspended in 100 \(\mu\)l of Tris-EDTA (TE) buffer, pH 8.0. Then, 11 \(\mu\)l of 10X protein K buffer (0.1 M Tris, pH 7.8, 50 mM EDTA, 5% SDS) and 1 \(\mu\)l of 20 \(\mu\)g/\(\mu\)l proteinase K were added and incubated at 45°C for 2 hour. DNA was extracted with phenol/chloroform, precipitated with ethanol, and the final DNA pellet was dissolved in 20 \(\mu\)l of TE buffer.

Polymerase chain reaction (PCR) amplications were done in 50 \(\mu\)l of 1 X PCR buffer containing DNA, 0.5 \(\mu\)M primers (forward primer: 5’ CCT TGA TGC CTC CAA CAA CT 3’ and backward primer: 5’ GGT GGG AGC TGA GAA GAC AG 3’ corresponding to nucleotides N-1043 to N-1026 and N-718 to N-698 of the rAGT gene promoter (47) or forward primer: 5’ TTC TGG GGC ATC CAT GAA ACT AC 3’ and backward primer: 5’ AGC ATT TGC GGT GCA CGA TGG AG 3’ corresponding to nucleotides N-808 to N-830 and N-1120 to N-1098 of the rat \(\beta\)-actin (48), respectively), 40 \(\mu\)M of each deoxynucleotide triphosphate, 1.5 mM MgCl2 and 1 unit of Tag DNA polymerase (InVitrogen, Inc.). The PCR products were resolved on 2% agarose gel and transferred onto a Hybond XL nylon membrane (Amersham-Pharmacia Biotech). Digoxigenin-labeled oligonucleotide 5’ CCT CCC TTC CCG GCC CCC TTC TCT TTC TAG T 3’, and 5’ CCA ACT CTC TTG CTA AAC AGA-3’ corresponding to nucleotides N-882 to N+855 of the rAGT gene promoter (47) and intron 4 nucleotide N-56 to N-77 of \(\beta\)-actin gene (48), respectively, 40 \(\mu\)M of each deoxynucleotide triphosphate, 1.5 mM MgCl2, and 1 unit of Tag DNA polymerase served to hybridize the PCR products on the membrane. After stringent washing, the membrane was detected with a digoxigenin luminescence kit (La Roche Biochemicals) and exposed to Kodak BMR film (Eastman Kodak Co., Rochester, NY, USA).
Mammalian Expression of Recombinant hnRNP K

Murine hnRNP K cDNA with flag-tag at the N-terminal in mammalian expression vector pRC/RSV (36) was transfected into IRPTCs with Lipofectamine according to the instruction manual provided by the supplier (Invitrogen Inc.). We optimized the DNA concentration for gene transfection at 2-3 µg per 0.5 to 1 x 10⁶ cells. Forty-eight h after transfection, total RNAs and nuclear proteins were isolated from IRPTCs and assayed for rAGT mRNA by RT-PCR (34, 35) or for flag-hnRNK protein by Western blotting, respectively.

Small Interfering RNA (siRNA) of hnRNP K

IRPTCs were transfected with 40 nM scrambled Silence™ Negative Control # 1 siRNA (Ambion Inc., Austin, TX, USA) or 40 nM siRNA for hnRNP K (Sense 5’ CCA GAU GUA AUG UUU UAG Utt 3’ and antisense 5’ ACU AAA ACA UUA CAU CUG Gtg 3’. Hnrpk siRNA ID 195920, Ambion Inc.) or 40 nM siRNA for hnRNP F (sense 5’ GCA UGG GAC ACC GGU AUA Utt 3’ and antisense 5’ AUU UAC CGG UGU CCC AUG Ctt 3’. HnRNP F siRNA ID 192101, Ambion Inc.). Transfections were accomplished by using siPORT Amine (Ambion Inc.) according to the manufacturer’s instructions. Total cellular RNA and protein were harvested at 48 h post-transfection and then analyzed for rAGT and β-actin mRNA, and hnRNP K protein expression by RT-PCR (35) and Western blotting, respectively.

Western Blotting for hnRNK K

Briefly, the cell pellets were lysed in 100 µl RIPA buffer, centrifuged, and 30 µg of the supernatants were subjected to 10% SDS-PAGE (44), then transferred onto a polyvinylidene difluoride membrane (Hybond-P, Amersham-Pharmacia Biotech). The membrane was initially blotted for anti-flag or anti-hnRNPK antibody (1:4,000 dilutions) and then re-blotted for anti-β-actin antibody (1:10,000 dilutions) and chemiluminescent developing reagent (La Roche Biochemicals). The relative densities of the hnRNPK and β-actin bands were quantified by computerized laser densitometry (ImageQuant software (version 5.1), Molecular Dynamics, Amersham-Pharmacia Biotech).

Pull-Down Assays

The cellular extracts were incubated with glutathion sepharose 4B beads with or without bound GST-hnRNPK or GST-hnRNP F (35) for 4 h at 4°C. The beads were washed 5 times with 1 ml of RIPA buffer, and the proteins were resolved by 10% SDS-PAGE (44) as described above. The membrane was blotted with anti-hnRNPK F (1:10,000 dilutions) or anti-hnRNPK antibody (1:4,000 dilutions) or anti-TBP antibody (1:1,000 dilutions) and developed with chemiluminescent developing reagent.

Co-immunoprecipitation Assays

IRPTC nuclear proteins were incubated with rabbit anti-hnRNPK F polyclonal antibody (10 µg) or normal rabbit IgG antibody (10 µg) for 4 h in RIPA buffer at 4°C. Then, protein-A-Sepharose beads were added and incubated for 1 h at 4°C. The beads were washed 5 times with RIPA buffer. The retained proteins were eluted with 2 x loading buffer and subjected to SDS-PAGE and Western blotting with anti-hnRNPK antibody.

Chloramphenicol Acetyl Transferase (CAT) Assay

The method of construction of the rAGT-CAT fusion gene, pOCAT/rAGT N-1498/+18 (fusion gene containing 1,498 nucleotides upstream of the transcription start site and 18 nucleotides of exon I fused with CAT reporter gene) and mutant pOCAT/rAGT N-1498/+18 with mutated IRE has been described previously (33, 35). Control plasmid or fusion gene was transfected into IRPTCs using lipofectamine (Invitrogen) according to methods described previously (33, 35). 48 h after transfection, the cells were harvested and assayed for CAT activity (33, 35).

To normalize the efficiency of transfection, 0.5 µg of pRSV/β-Gal [a vector with the Rous Sarcoma Viral enhancer/promoter fused to the bacterial β-galactosidase gene] was co-transfected with pOCAT/rAGT N-1498/+18 (33, 35). The plasmid pRSV/CAT [a vector with the Rous Sarcoma Viral enhancer/promoter fused to the CAT reporter gene] served as a positive control to monitor the efficiency of transfection of rAGT-CAT fusion gene. The level of transfection efficiency for pRSV/CAT in IRPTCs ranged from
60 to 90%, i.e., the percentage of conversion of $^{14}$C chloramphenicol to mono- and di-acetyl chloramphenicol. The transfection efficiency of pOCAT/rAGT N-1498/+18 in IRPTCs ranged from 25% to 35% compared with pRSV-CAT. The inter- and intra-assay coefficient variations of transfection for pOCAT/rAGT N-1498/+18 in IRPTCs were 25% and 12% ($N = 10$), respectively.

**Animals**

The streptozotocin (STZ)-induced diabetic Wistar rat model has been described previously (49). Briefly, adult male Wistar rats (200-250 g) obtained from Charles River Inc. (St-Constant, QC, Canada) were divided into 3 groups: 1. Vehicle-injected controls (10 mM sodium citrate buffer, pH 4.0); 2. Untreated STZ-induced diabetics (65 mg/kg of STZ dissolved in 10 mM sodium citrate buffer administered i.p. after overnight fasting); and 3. Treated STZ-induced diabetics (subcutaneous insulin implant 48 hours after STZ induction). Untreated and treated diabetic rats with blood glucose $>25$ mM and $<7$ mM, respectively, were studied. Blood glucose was monitored with a glucose analyzer (Accu-Check Compact, Roche Diagnostics, Laval, QC, Canada).

All animals were allowed free access to rat chow and water. All methods of animal care and sacrifice were approved by the Animal Care Committee of the CHUM.

**Isolation of Rat RPTs**

Two weeks after the induction of diabetes, the rats were anesthetized and euthanized [Control and treated rats were euthanized at the same time point]. Kidneys were removed immediately for proximal tubule isolation. The renal cortex was separated from the medulla and minced under sterile conditions. Proximal tubules were isolated by Percoll gradient (50) with slight modifications (49). Proximal tubular cells were characterized by their histological appearance (50). A highly-purified preparation of proximal tubules (>97% by microscopy) with >95% viability (determined by trypan blue exclusion) was obtained. Aliquots of freshly-isolated proximal tubules from individual rats were immediately used for total RNA and protein isolation.

**Statistical analysis**

Three to 5 separate independent experiments were performed per protocol, and each treatment group was run in duplicate. The data were analyzed by 1-way ANOVA and the Bonferroni test. A probability level of $p \leq 0.05$ was regarded as statistically significant.

**RESULTS**

**Identification of 70-kDa IRE-Binding Proteins (BPs) in IRPTCs.** Figure 1A shows the staining of nuclear proteins after 2-D electrophoresis. Southwestern blotting of IRE-BPs after 2-D electrophoresis is displayed in Figure 1B. Positive spots with apparent MW of 70 and 48 kDa were cut out and subjected to MALDI-MS. The MS results of 70-kDa proteins are displayed in Figure 2. The 2 spots with an apparent MW of 70 kDa were identified as a common protein (Accession number NM_057141). Database analysis revealed that they are identical to the rat hnRNP K cDNA sequence reported by Ito et al. (51). The 2 spots with an apparent MW of 48 kDa were identified as 46 kDa hnRNP F (35) (data not shown).

To confirm the authenticity of rat hnRNP K revealed by Southwestern blotting, we stripped the radioactivity from the membrane and re-blotted it with rabbit polyclonal antiserum against hnRNP K, as shown in Figure 1C. It is apparent that the proteins interacting with anti-hnRNP K were superimposable, with positive signals detected by Southwestern blotting, as seen in Figure 1B. These data confirm that the 70-kDa proteins that interact with ANG-IRE are identical to hnRNP K.

**GMSA of Radioactively-Labeled rAGT-IRE DNA Fragment with GST-hnRNP K Fusion Protein(s).** Bacterially-expressed recombinant hnRNP K proteins were employed to study the interaction of rAGT-IRE with hnRNP K. One major band appeared with retarded mobility with labelled rAGT-IRE by employing GST-hnRNP K fusion protein (Figure 3A). No slowly-migrating band was observed when the labeled DNA was incubated with GST (bacterial extract of empty vector pGex 4T-3). The addition of an unlabeled rAGT-IRE was effective in competing with the binding of labeled rAGT-IRE DNA to the fusion proteins(s) (100-fold molar excess of unlabeled DNA fragment), but not the unlabeled DNA.
fragment of hGAPDH-IRE, rat glucagon-IRE, and the Sp1 consensus sequence (Figure 3A) or mutants (M1 and M2) of rAGT-IRE (Figure 3B). GST-hnRNP K bound specifically to wide type rAGT-IRE but not to mutant rAGT-IRE (Figure 3C).

ChIP Analysis of HnRNP K Interactions with Gene Loci. ChIP assays were used to test if hnRNP K interacts with the IRE of the rAGT gene promoter in vivo. Figure 4 displays the PCR product of pulled-down DNA with primers specific to rAGT and β-actin gene. A ~300-bp DNA fragment was apparent in input control cells and cells that were treated with the cross-linking agent and without blocking peptide (lanes 2 and 4). In contrast, no PCR product was generated in cells without treatment with hnRNPK antibody (lane 3) or in the presence of blocking peptide (lane 5). PCR product specific to β-actin was also apparent in input control cells (lane 2) but not in cells that were treated with or without hnRNPK antibody in the absence or presence of hnRNPK blocking peptide. These results validate the interaction of hnRNPK with rAGT gene loci in vivo.

Effect of hnRNPK on rAGT mRNA Expression in IRPTCs. It is apparent that hnRNPK protein levels in IRPTCs transiently transfected with pRSV/hnRNPK were significantly higher (>2-fold increase, p<0.005) than those in control pRC/RSV-transfected IRPTCs (Figure 5A) by Western blotting. In contrast, rAGT mRNA expression was significantly lower (50% decrease, p<0.005) in pRSV/hnRNPK K-transfected cells than those in pRC/RSV-transfected cells analyzed by RT-PCR (Figure 5B). These results demonstrated that hnRNPK K inhibits rAGT mRNA expression in IRPTCs.

High glucose stimulated (>3-fold increase, p<0.01) and insulin inhibited rAGT mRNA expression in IRPTCs as shown in Figure 6A. In contrast, hnRNPK K overexpression prevented the stimulatory effect of high glucose on rAGT mRNA expression in IRPTCs (Figure 6B). These data suggest that hnRNPK K modulates high glucose stimulation of rAGT gene expression in IRPTCs.

Effect of siRNA of hnRNPK and hnRNPF on rAGT mRNA Expression in IRPTCs. Transient transfer of siRNA of hnRNPK and hnRNPF suppressed respective hnRNPK and hnRNPF expression (Figure 7A) but enhanced rAGT mRNA expression in IRPTCs (Figure 7B). In contrast, transient transfer of negative scrambled silencer had no effect on hnRNPK or hnRNPF protein and rAGT mRNA expression in IRPTCs. These data further support that both hnRNPF and hnRNPK modulate rAGT mRNA expression in IRPTCs.

Effect of hnRNPK on rAGT Gene Promoter Activity. Like hnRNPF (35), co-transfection with hnRNPK K significantly suppressed pOCAT-rAGT fusion gene promoter activity (Figure 8) but had no effect on mutant pOCAT-rAGT fusion gene promoter activity. These studies further confirm the notion that hnRNPK K modulates rAGT gene expression at the transcriptional level via binding to IRE.

Interaction of hnRNPK with hnRNPF in Pull-Down and Co-immunoprecipitation Assays. Figure 9A shows that GST-hnRNPK K was pulled down with hnRNPF from IRPTCs. Vice versa, GST-hnRNPF was pulled down with hnRNPK K from IRPTCs (Figure 9B). GST by itself had no effect. We also confirmed the interaction between hnRNPF and hnRNPK by co-immunoprecipitation (Figure 9C). These data demonstrate that hnRNPK K could form a heterodimer with hnRNPF.

Effect of Co-transfection of hnRNPK and hnRNPF on rAGT mRNA Expression in IRPTCs. Figure 10A shows that the ATG mRNA expression is significantly lowered in IRPTCs that had been stably transfected with pcDNA 3.1/hnRNPF (35) as compared to cells that had been stably transfected with control plasmid pcDNA 3.1. Transient transfection with hnRNPK K further suppressed rAGT mRNA expression in a dose-dependent manner in IRPTCs that had been stably transfected with pcDNA 3.1/hnRNPF (Figure 10B). These studies support the notion that hnRNPK K and hnRNPF could act additively to modulate endogenous rAGT gene expression in IRPTCs.

Effect of High Glucose and Insulin on hnRNPK Expression In Vitro and In Vivo. Figures 11A and 11B show the results of respective Southwestern and Western blot analysis of IRPTC...
nuclear extracts for hnRNP K incubated in normal glucose (5 mM D-glucose plus 20 mM D-mannitol) or high glucose (25 mM D-glucose) medium in the absence or presence of insulin (10^{-7} M). It is apparent that high glucose levels enhanced and insulin suppressed hnRNP K expression in IRPTCs in vitro.

Similarly, studies in vivo revealed that hyperglycemia upregulated hnRNP K expression in diabetic rat RPTs, and insulin treatment normalized hnRNP K to non-diabetic levels (Figure 12). These data demonstrate that hyperglycemia and insulin regulate hnRNP K expression in diabetic rat RPTs.

**DISCUSSION**

The present studies identified hnRNP K as one of the nuclear proteins that binds to IRE of the rAGT gene promoter and inhibits rAGT gene expression in IRPTCs.

We reported recently that by employing a combination of proteomics and Southwestern blotting, 46-kDa hnRNP F was identified as the 48-kDa nuclear protein that binds to IRE of the rAGT gene promoter and inhibits rAGT gene expression in IRPTCs (35).

The present studies aimed to identify the molecular structure of the 70-kDa nuclear protein with the same approach as with 46-kDa hnRNP F. It is apparent that multiple IRPTC proteins are resolved by 2-D electrophoresis. A second gel run simultaneously with the first gel for Southwestern blotting demonstrated 2 positive spots with an apparent MW of 70 kDa, and pI of 5.0 to 6.0 closely matched the stained protein spots on the first gel. After tryptic digestion and MALDI-MS, these 2 spots were found to match the partial amino acid sequence deduced from the hnRNP K sequence as reported by Ito et al. (51) (Accession number NM_057141). Using specific rabbit antiserum against hnRNP K, we confirmed the identity of hnRNP K on the membrane by Southwestern blotting. Western blotting revealed positive signals superimposed on the positions of the 2 spots detected by Southwestern blotting. The reason why hnRNP K is present in 2 different forms (same apparent MW but different pI) is presently unclear. One possibility is that these proteins might be isoforms or variants with different phosphorylated forms. We have further confirmed that the 48-kDa species by MALDI-MS analysis is 46-kDa hnRNP F as we have reported previously (35, data not shown).

HnRNP K was identified as one of these hnRNPs that binds cytidine-rich elements (52-54). HnRNP K is encoded by 1 gene and can be alternatively spliced to at least 4 isoforms with deduced MW in the range of 50-51 kDa but in SDS-PAGE, hnRNP K has an apparent MW of 65 kDa (55). HnRNP K has been localized in the nucleus, cytoplasm and mitochondria, and implicated in chromatin remodeling, transcription, splicing and translation processes (see review by Bomsztyk et al. (56, 57)). HnRNP K binds single-stranded (ss) and double-stranded (ds) DNA motifs (CT element, 5’d-TCCC) within the promoter of c-myc, c-src and c-fos gene, and complexes with Sp1 and TATA-binding protein (TBP) to modulate gene transcription (58-61). HnRNP K also represses the transcription of thymidine kinase (62), the neuronal nicotinic acetylcholine receptor β4 subunit (63), and osteocalcin (64). Thus, hnRNP K is a multifunctional protein that interacts with DNA, RNA, transcriptional and translational molecules to alter the in vivo rate of gene transcription and translation (either stimulation or repression).

To demonstrate that hnRNP K interacts with rAGT-IRE, we expressed hnRNP K in a bacterial system. Our GMSAs revealed that labeled rAGT-IRE binds to GST-hnRNP K fusion protein. The addition of unlabeled rAGT-IRE DNA effectively displaced labeled rAGT-IRE at or greater than a 100-fold molar excess of unlabeled DNA, whereas mutant rAGT-IRE was not effective. Similarly, unlabeled hGAPDH-IRE, rat glucagon-IRE, and the Sp1 consensus sequence were not effective in displacing labeled rAGT-IRE. Moreover, GST-hnRNP K did not bind mutant rAGT-IRE. These studies indicate that rAGT-IRE binds specifically to hnRNP K. We did not observed, however, a supershift of labeled rAGT-IRE binding with hnRNP K fusion protein by several different batches of anti-hnRNP K antibodies generated in our laboratory (K.B.) or obtained from Dr. Gideon Dreyfuss (data not shown). At present, we have no explanation for this observation. One possibility is that the binding affinity of antibodies is not high enough. The second possibility is that the structure of hnRNP K might hinder the binding with anti-hnRNP K

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antibodies. Clearly, more experiments are needed to clarify this observation.

Most convincingly, our ChIP assays revealed that hnRNP K interacts with rAGT gene promoter loci. Taken together, these data unequivocally demonstrate that hnRNP K binds to rAGT-IRE.

Interestingly, transient transfection of hnRNP K inhibits rAGT gene expression in IRPTCs, and stable transfection of hnRNP K cDNA prevented the stimulatory effect of high glucose on rAGT mRNA expression in IRPTCs. In contrast, suppression of hnRNP K expression by siRNA enhances rAGT gene expression in IRPTCs. To the best of our knowledge, this is the first report that hnRNP K could modulate rAGT gene expression in vitro. The negative effect of hnRNP K is similar to that of hnRNP F on rAGT gene expression (35). At present, the molecular mechanism(s) of hnRNP K action on rAGT mRNA expression is not known. One possibility is that hnRNP K behaves like a negative transacting protein and inhibits the binding of other positive transacting factor(s) to TBP and RNA polymerase II, subsequently attenuating rAGT gene expression. This possibility is supported by the findings including ours (unpublished results) that the hnRNP K molecule binds directly to TBP and, therefore, may inhibit the basal transcription machinery (37, 53). The second possibility is that hnRNP K overexpression inhibits the formation of an activating transcriptional complex on the promoter and subsequently represses rAGT gene expression. This possibility is supported by the studies of Du et al. (63) and Da Silva et al. (65), showing that hnRNP K inhibits Sp1 binding to the promoter of the gene encoding the β₄ subunit of the nicotinic acetylcholine receptor and CD 43 gene promoter, respectively. The third possibility is that hnRNP K could recruit unidentified repressor molecules and subsequently repress rAGT gene expression. This possibility is supported by previous studies demonstrating that hnRNP K could bind the murine repressor Zik1 (66). Finally, it is also possible that hnRNP K could form an heterodimer with hnRNP F (a hypothetical stable complex) and then bind to rAGT-IRE and subsequently attenuate rAGT gene expression. This possibility is supported by the findings that hnRNP K could form an heterodimer or trimer with hnRNP A1/or hnRNP C to inhibit human thymidine kinase promoter (62). Indeed, this possibility is further supported by our findings that hnRNP K was pulled down and co-immunoprecipitated with hnRNP F. Co-transfection of hnRNP K and hnRNP F further suppressed rAGT mRNA expression. Clearly, more work is needed along these lines to elucidate the mechanism(s) of action of hnRNP K on rAGT gene expression in IRPTCs.

Finally, our studies revealed that high glucose stimulated and insulin inhibited hnRNP K expression in IRPTCs in vitro and in diabetic rat kidneys in vivo. These data are consistent with our previous studies in IRPTCs in vitro (34). To the best of our knowledge, this is the first report that hnRNP K could be modulated by high glucose and insulin in kidney proximal tubular cells in vitro and in vivo. The exact physiological role(s) of hnRNP K on renal rAGT gene expression is unknown. Our preliminary data revealed that hnRNP K mRNA expression is significantly lower in RPTs of spontaneously diabetic BioBreeding (BB) rats compared to non-diabetic controls, whereas rAGT mRNA expression is significantly higher in RPTs of diabetic BB rats (unpublished results). These studies indicate that hnRNP K is an endogenous suppressor and may play an important role in counterbalancing AGT gene overexpression in high glucose in vivo. Studies are ongoing in our laboratory along this line.

In summary, we have established, by a combination of Southwestern blotting and proteomics, that hnRNP K is a nuclear protein that binds to rAGT-IRE and inhibits rAGT gene expression in IRPTCs. It appears that high glucose and insulin regulate hnRNP K expression in kidney proximal tubular cells. Our studies raise the possibility that hnRNP K expression may play a role in counterbalancing high-glucose stimulation of rAGT gene expression and in modulating local intrarenal RAS activation. Dysregulation of hnRNP K expression may contribute to renal injury in diabetes via altered local intrarenal RAS activation.
APPENDIX

Abbreviations used in this article: ACE, angiotensin-converting enzyme; rAGT, rat angiotensinogen; Ang II, angiotensin II; CAT, chloramphenicol acetyl transferase; ChIP, chromatin immunoprecipitation; DN, diabetic nephropathy; ESRD, end-stage renal disease; GMSA, gel mobility shift assay; hnRNP F, heterogenous nuclear ribonucleoprotein F; hnRNP K, heterogenous nuclear ribonucleoprotein K; IP, immunoprecipitation; IEF, isoelectrofocusing; IRE, insulin-responsive element; IRE-BP, IRE-binding protein; IRPTCs, immortalized renal proximal tubular cells; kDa, kiloDalton; MALDI, matrix-assisted laser desorption/ionization technique; MS, mass spectrometry; MW, molecular weight; PCR, polymerase chain reaction; RAS, renin-angiotensin system; RPTCs, renal proximal tubular cells; RT-PCR, reverse transcriptase-polymerase chain reaction; RPTs, renal proximal tubules; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ss, single-stranded; siRNA, small interfering RNA; STZ, streptozotocin; 2-D, 2 dimensional; TBP, TATA-binding protein; TGF-β1, transforming growth factor-beta 1.

ACKNOWLEDGEMENTS

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LEGENDS

Figure 1: Rat AGT IRE-BPs detected by 2-D electrophoresis, Southwestern and Western blottings. (A) Rat IRPTC nuclear extracts were subjected to 2-D electrophoresis and then stained with Coomassie Brilliant Blue R-250. M: Amersham-Pharmacia Biotech’s rainbow molecular mass markers. N.E.: Rat IRPTC nuclear extracts without IEF. (B) Southwestern blotting analysis of AGT IRE-BPs from IRPTC nuclear proteins after 2-D electrophoresis. After 2-D electrophoresis, the nuclear proteins were transferred onto a Hybond C-extra membrane, hybridized with radioactively-labeled concanmeric AGT-IRE (N-878/N-864), washed, and subjected to autoradiography. The arrow heads indicate the proteins that were determined to be hnRNP K by later MS. The rectangle denotes the 70-kDa proteins that were subjected to MS, their identities were determined as 65-kDa hnRNP K. (C) Western blotting of the 2-D electrophoresis membrane from panel B using anti-hnRNP K antibody.

Figure 2: MS analysis of proteins detected by Southwestern blotting. Spot 1 was isolated from 2-D gel and then subjected to trypsin digestion and MALDI-MS analysis. The peptide sequences homology with hnRNP K identified by MALDI-MS and database search were shown in BOLD letters. The sequence coverage of hnRNP K reached 14%. Similar results were obtained from spot 2 (not shown).

Figure 3: GMSA of radioactively-labeled rAGT-IRE DNA fragment with GST-hnRNP K fusion protein(s). The labeled DNA probe (0.1 pmol) was incubated with GST (5 µg) or GST-hnRNP K fusion protein(s) (1.5 µg) in the presence of 1 µg of poly dl-dC. Competition with excess hGAPDH-IRE, rat glucagons-IRE, Sp1 consensus sequence, and monomeric wt rAGT-IRE is shown in lanes 4 and 5, lanes 6 and 7, lanes 8 and 9, and lanes 10-11, respectively (Figure 3A). Similarly, competition with 100- and 300-fold molar excess of unlabeled 3 x rAGT-IRE, rAGT-IRE mutants (M1, M2, M3 and M4), and monomeric wild type (wt) rAGT-IRE is shown in lanes 4 and 5, lanes 6 and 7, lanes 8 and 9, lanes 10-11, lanes 12-13, and lanes 14 and 15, respectively (Figure 3B). Only concanmeric wide type 3 x rAGT-IRE could bind to GST-hnRNP K but not mutant 3 x rAGT-IRE is shown in lanes 2 and 3, and lanes 4 and 5, respectively (Figure 3C).

Figure 4: ChIP analysis with anti-hnRNP K antibody. The cells were lysed, and nuclei were isolated and then sonicated. HnRNP K was immunoprecipitated without (-, lane 3) or with (+, lane 4) anti-hnRNP K antibody or in the presence of blocking peptide (lane 5). Complexes were eluted, cross-linking was reversed, and purified DNA was used as a template in PCR with primers specific to the rat AGT gene promoter and β-actin gene. DNA was separated by agarose gel electrophoresis and visualized. Then, the DNA was transferred onto a Hybond XL nylon membrane and hybridized with a DIG-labeled internal DNA probe.

Figure 5: Effect of transient transfection of hnRNP K cDNA on AGT mRNA expression in IRPTCs. A. Western blotting of hnRNP K protein in IRPTCs transiently transfected with pRC/RSV or pRSV/hnRNP K. B. RT-PCR analysis of endogenous rAGT and β-actin mRNA expression in IRPTCs transiently transfected with pRC/RSV or pRSV/hnRNP K. Twenty-four h after gene transfection, the cells were cultured for 24 h in 5 mM DMEM containing 5% FBS. Then, the cells were harvested and assayed for hnRNP K and rAGT mRNA by Western blotting and RT-PCR assay, respectively. The relative levels of hnRNP K protein and AGT mRNA were compared with β-actin protein and β-actin mRNA, respectively. The hnRNP K and AGT mRNA level in pRC/RSV-transfected cells represents the control level (100%). The results were expressed as means ± SD of 3
independent experiments and each treatment group was assayed in triplicate (**p ≤ 0.005).

**Figure 6:** Over-expression of hnRNP K prevented the high glucose effect on AGT mRNA expression in IRPTCs. RT-PCR analysis of endogenous rAGT mRNA expression in IRPTCs stably tranfected with control plasmid pRC/RSV (A) or pRSV/hnRNP K (B). Twenty-four h after synchronization, the cells were cultured for 24 h in 5 mM DMEM plus 20 mM D-glucose, 25 mM D-glucose in the absence or presence or insulin (10^{-7} M) containing 1% depleted FBS. Then, the cells were harvested and assayed for rAGT mRNA by RT-PCR. The relative levels of ANG mRNA were compared with β-actin mRNA. The AGT mRNA level in 5 mM D-glucose medium represents the control level (100%). The results were expressed as means ± SD of 3 independent experiments and each treatment group was assayed in duplicate (*p ≤ 0.05; **p ≤ 0.01; NS, not significant).

**Figure 7:** Effect of siRNA of hnRNP F and hnRNP K on rAGT mRNA expression in IRPTCs. 48 h after transfection, the cells were harvested and assayed for hnRNP F or hnRNP K protein (A) and AGT mRNA expression (B) by Western blotting and RT-PCR, respectively. The level of hnRNP F or hnRNP K and AGT mRNA expression was compared with β-actin protein and mRNA, respectively. The results were expressed as means ± SD of 3 independent experiments and each treatment group was assayed in duplicate (**p ≤ 0.01; ***p ≤ 0.005; NS, not significant).

**Figure 8:** Effect of hnRNP K on rAGT gene promoter activity in IRPTCs. 48 h after transfection of pRSV/CAT, wild-type pOCAT/rAGT N-1498/+18, or mutant pOCAT/rAGT N-1498/+18 with or without hnRNP F or hnRNP K cDNA, the cells were harvested and assayed for CAT activity. Relative activity in cells transfected with 2.5 µg of rAGT N-1498/+18 or its mutant was given a relative value of 100% (control). Each point represents the mean ± S.D. of 3 independent experiments and each treatment group was assayed in duplicate (**p ≤ 0.01; ***p ≤ 0.005; NS, not significant).

**Figure 9:** Interaction of hnRNP K with hnRNP F. Equal amounts of purified GST or GST-hnRNP K (A) or GST-hnRNP F (B) bound to glutathione beads were mixed with IRPTC extract and incubated for 4 h at 4°C. After extensive washes, the proteins were resolved by 10% SDS-PAGE, transferred to membrane, and blotted with anti-hnRNP F antibody (A) or anti-hnRNP K (B). IRPTC nuclear proteins (N.P.) were incubated with 10 µg of anti-hnRNP F antibody (anti-F) or normal rabbit IgG. The immunocomplex was precipitated by protein-A-Sepharose and washed 5 times with RIPA buffer. The proteins were eluted and subjected to 10% SDS-PAGE, transferred to membrane, and blotted with anti-hnRNP K antibody (C). Similar results were obtained from two other experiments (W.B., Western blotting).

**Figure 10:** Effect of co-transfection of hnRNP K and hnRNP F on rAGT mRNA expression in IRPTCs. (A) Comparison of the AGT mRNA level in IRPTCs that had been stably transfected with plasmid pcDNA 3.1 or pcDNA 3.1/hnRNP F (35). (B) pRSV/hnRNP K was transiently transfected into IRPTCs that had been stably transfected with pcDNA 3.1/hnRNP F. Twenty-four h after transfection, the cells were harvested and assayed for rAGT mRNA by RT-PCR. The relative levels of AGT mRNA were compared with β-actin mRNA. The AGT mRNA level in IRPTCs that had been transiently transfected with 2 µg of control plasmid pRC/RSV represents the control level (100%). The results were
expressed as means ± SD of 3 independent experiments and each treatment group was assayed in duplicate (**p ≤ 0.01; ***p ≤ 0.005, NS, not significant).

Figure 11: Effect of high glucose and insulin on hnRNP K expression in IRPTCs analyzed by Southwestern and Western blotting. The cells were incubated in 5 mM plus 20 mM D-mannitol, 25 mM D-glucose or 25 mM D-glucose plus insulin (10^{-7} M) and 5% FBS for 24 h. Then, the cells were harvested and nuclear proteins (100 µg) were electrophoresed on SDS-PAGE [(4-20% (W/V) gradient gel], transferred onto a polyvinylidene difluoride membrane, analyzed by Southwestern blotting with radioactive rAGT-IRE probe (A) and then by Western blotting with rabbit polyclonal antibodies against hnRNP K (B) and ECL-chemiluminescent developing reagent. The same membrane was also hybridized with antibodies against β-actin control. The relative densities of the hnRNP K bands were compared with the β-actin control. The hnRNP K level expressed in IRPTCs in 5 mM glucose medium was considered to be the control (100%). Each bar represents the mean ± SD of 3 independent experiments and each treatment group was assayed in duplicate (*p ≤ 0.05, **p ≤ 0.01).

Figure 12: HnRNP K expression in non-diabetic and diabetic rat RPTs. After 2 weeks of STZ-induced diabetes with or without insulin supplementation, RPTs were collected, extracted for proteins and assayed for hnRNP K and β-actin levels by Western blotting. The relative levels of hnRNP K were normalized with β-actin. HnRNP K levels in non-diabetic rats were considered as 100%. Each point represents the mean ± SD of 5 rat RPTs. ***, p ≤ 0.001.
Figure 1
Wei et al.

A. Protein staining  
B. Southwestern blotting  
C. Western blotting
Matched peptides show in **Bold**

1  METEQPEETF PNTETNGEF GKRPAEDNEEE QAFKRSRNTD EMVEIRILLQ
51  SKNAGAVIGK GGKNIKALRT DYNASVSVPD SSGPERILSI SADIELTIGET
101  IKKIPTLEE GLQLPSPTAT SQLPLESDAV ECLNYQHYKG SDFDCERLL
151  **IHOSLAGGII** GVKGAKIKEL RENTOTTIKL FOECCPHSRD RVVLIGGKPFD
201  RVVECIKIIL DLIESPIKG RAQPYPDNPY DETYDYGGFT MMTFDRRGRP
251  VGFEMRGRGG FDRNPPGRGG REMPPSRRDY DDMSPRGPPP PPPPGRGGRG
301  GSRARNLFLP **PPPP**RGGDLD MAYDRGRPG DRYDGMVGFS ADETWSAID
351  **TWSPEWOMA** YEPOGGSGYD YSYAGGRGSY GDLLGPIITT QVTIFKDLAG
401  SIIKGGGQRI KQIRHESGAS IKIDEPLEGS EDRIITITGT QDQIQRNAQYL
451  LQNSVKQYSG KFF

**Match to:** Rat hnRNP K  (*NM_057141*)

**Nominal mass (Mr):** 51230;  **Calculated pI value:** 5.39

**Sequence Coverage:** 14%
Figure 3
Wei et al.
Figure 4
Wei et al.

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→ PCR of rAGT-IRE
→ PCR of β-actin
Figure 5  
Wei et al.

A.

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B.

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### Figure 6
Wei et al.

#### A. pRC/RSV

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![Image of Western blots for A. pRC/RSV](image)

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#### B. pRSV/hnRNP K

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![Image of Western blots for B. pRSV/hnRNP K](image)

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#### Graphs

**A. pRC/RSV**

- Ratio of rAGT/β-Actin mRNA (% of control, 100%)
- D-Glucose: 5 mM, 25 mM, 25 mM + Ins, D-Mannitol: 20 mM

**B. pRSV/hnRNP K**

- Ratio of rAGT/β-Actin mRNA (% of control, 100%)
- D-Glucose: 5 mM, 25 mM, 25 mM + Ins, D-Mannitol: 20 mM

H.S. = Highly Significant, N.S. = Not Significant
Figure 7
Wei et al.

A.

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<tr>
<td>β-actin mRNA</td>
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</table>

![Graphs showing the ratio of protein and mRNA expression](image)

![Bar graphs showing the ratio of rAGT/β-actin mRNA](image)
Figure 9
Wei et al.

A. Input

Beads

- GST
- GST-hnRNP K

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<tr>
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<th>2</th>
<th>3</th>
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<td>+</td>
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<td>GST-hnRNP K</td>
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kDa

160 105 75

Anti-hnRNP F (W.B.)

50 35

B. Input

Beads

- GST
- GST-hnRNP F

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<td>GST</td>
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<tr>
<td>GST-hnRNP F</td>
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kDa

160 105 75

Anti-hnRNP K (W.B.)

75 50 35

C. Input

Beads

N.P.  I.P.  IgG  Anti-F

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<tr>
<td>I.P.</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>IgG</td>
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<td>-</td>
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</tr>
<tr>
<td>Anti-F</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>
A.

pcDNA3.1 pcDNA3.1/hnRNP F

rAGT mRNA

β-actin mRNA

B.

pRSV/hnRNP K (µg) - 0.5 1 1.5 2
pRC/RSV (µg) 2 1.5 1 0.5 -

rAGT mRNA

β-actin mRNA

Figure 10
Wei et al.
**Figure 11**
Wei et al.

### A  Southwestern blotting

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<tr>
<td>Insulin (1 X 10^{-7} M)</td>
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### B  Western blotting

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<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 12
Wei et al.

![Graph showing ratio of hnRNP K/β-actin protein](image)

- Non-diabetic
- STZ-diabetic
- STZ-diabetic + Ins.

Legend:
- hnRNP K
- β-actin

Bar graph indicating the ratio of hnRNP K/β-actin protein (% of control, 100%) among different groups.
Heterogenous nuclear ribonucleoprotein K modulates angiotensinogen gene expression in kidney cells
Chih-Chang Wei, Shao-Ling Zhang, Yun-Wen Chen, Deng-Fu Guo, Julie R. Ingelfinger, Karol Bomsztyk and John S.D. Chan

*J. Biol. Chem.* published online July 12, 2006

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