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Balázs Szamosfalvi
Henry Ford Health, bszamos1@hfhs.org

Pedro Cortes
Henry Ford Health

Rebecca Alviani
Henry Ford Health

Kenichiro Asano
Henry Ford Health

Bruce L. Riser
Henry Ford Health

See next page for additional authors

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Authors
Balázs Szamosfalvi, Pedro Cortes, Rebecca Alviani, Kenichiro Asano, Bruce L. Riser, Gerald Zasuwa, and Jerry Yee

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Putative subunits of the rat mesangial K_{ATP}: A type 2B sulfonylurea receptor and an inwardly rectifying K^+ channel

Balázs Szamosfalvi, Pedro Cortes, Rebecca Alviani, Kenichiro Asano, Bruce L. Riser, Gary Zasuwa, and Jerry Yee

Department of Medicine, Division of Nephrology and Hypertension, Henry Ford Hospital, Detroit, Michigan, USA

Putative subunits of the rat mesangial K_{ATP}: A type 2B sulfonylurea receptor and an inwardly rectifying K^+ channel.

Background. Sulfonylurea agents exert their physiological effects in many cell types via binding to specific sulfonylurea receptors (SUR). SUR couple to inwardly-rectifying K^+ channel (Kir6.x) to form tetradimeric ATP-sensitive K^+ channels (K_{ATP}). The SUR subunits confer ATP-sensitivity on K_{ATP} and also provide the binding sites for sulfonylureas and other pharmacological agents. Our previous work demonstrated that the exposure of mesangial cells (MC) to sulfonylureas generated profound effects on MC glucose uptake and matrix metabolism and induced heightened cell contractility in association with Ca^{2+} transients. Because these responses likely resulted from the binding of sulfonylurea to a mesangial SUR2, we subsequently documented [^{3}H]-glibenclamide binding to MC and the gene expression of several mesangial SUR2 transcripts. From these data, we inferred that MC expressed the components of a mesangial K_{ATP} and sought to establish their presence in primary MC.

Methods. To obtain mesangial SUR2 cDNA sequences, rapid amplification of cDNA ends (RACE) was utilized. DNA sequences were established by the fluorescent dye termination method. Gene expression of mesangial SUR2 and Kir6.1/2 was examined by reverse transcription polymerase chain reaction (RT-PCR) and Northern analysis. SUR2 proteins were identified by immunoblotting of mesangial proteins from membrane-enriched fractions with polyclonal antiserum directed against SUR2.

Results. RACE cloning yielded two mesangial SUR2 cDNAs of 4.8 and 6.7 kbp whose open reading frames translated proteins of 964 and 1535 aa, respectively. Using probes specific to each cDNA, the presence of a unique, 5.5 kbp serum-regulated mesangial SUR2 splice variant was established. The sequence of this mesangial SUR2 (mcSUR2B) shares identity with the recently cloned rat SUR2B (rSUR2B), but, in comparison to rSUR2B, is truncated by 12 exons at the N-terminus where it contains a unique insert of 16 aa. Immunoblotting studies with anti-SUR2 antiserum demonstrated SUR2 proteins of 108 and 170 kD in membrane-enriched fractions of MC protein extracts. Complementary studies showed abundant gene expression of Kir6.1, thereby establishing gene expression of both components of K_{ATP}.

Conclusions. Based upon analogy to vascular smooth muscle cells (VSMC), there are at least two putative mesangial K_{ATP} that most likely represent hetero-octamers, comprised of either rSUR2B or mcSUR2 in complex with Kir6.1. Our results define the mesangial SUR2B as the possible first link in a chain of cellular events that culminates in MC contraction and altered extracellular matrix metabolism following exposure to sulfonylureas. In addition, our results serve as the basis for the future elucidation of the electrophysiologic characteristics of the mesangial K_{ATP} and the study of endogenous regulators of mesangial cell contractility.

Key words: mesangial cell contraction, hypoglycemia, cell contraction, potassium channels, glucose uptake, SUR2B, Kir6.1.

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Sulfonylureas such as tolazamide and glibenclamide are oral hypoglycemic agents that are frequently utilized during the treatment of type 2 diabetes mellitus [1]. The elucidation of the mechanism by which these agents augment pancreatic β cell insulin secretion via their high affinity type-1 sulfonylurea receptors (SUR1) has impelled a burgeoning field of investigation into pancreatic and non-pancreatic SUR [2]. These receptors are members of the cystic fibrosis transmembrane regulator (CFTR)/multidrug resistance protein (MRP) subfamily that is subsumed within the adenosine 5′-triphosphate (ATP)-binding cassette (ABC) superfamily [3, 4]. The two SUR genes, *SUR1* and *SUR2*, are encoded on separate chromosomes and have been respectively designated, ABCC8 and ABCC9 [5]. The SUR are predicted to have 17 transmembrane-spanning helices organized into three domains designated TM0, TM1, and TM2, separated by two intracytoplasmic loops labeled NBF1 and NBF2, each containing nucleotide-binding motifs. The SUR differ in their affinity for sulfonylureas and in their tissue distribution. SUR1 is a high-affinity receptor expressed by pancreatic β cells and brain, while the low-affinity SUR2 is more ubiquitously expressed [2]. There are two major alternatively spliced SUR2 variants, SUR2A and SUR2B, and the former is chiefly found in heart and skeletal muscle, while the latter is localized to brain,

heart, liver, kidney, intestine, and vascular smooth muscle [6, 7].

The ATP-sensitive K^+ channels (K_{ATP}) are heterooctamers of two subunits, a SUR and a weak inwardly-rectifying K^+ channel (Kir6.x) that associate with each other in a stoichiometry of 4:4. Ion-channel closure is mediated by sulfonylurea binding or by increases of the cytoplasmic ATP:ADP ratio. In K_{ATP}, SUR1 or SUR2 regulates the Kir6.x ion pore to which it is coupled [8–16]. The prototypical β cell K_{ATP} is a tetradimer of SUR1 and Kir6.2, whereas the cardiac and vascular smooth muscle K_{ATP} are represented by $(SUR2A:Kir6.2)_4$ and $(SUR2B:Kir6.1/2)_4$, respectively [17–24]. In previous studies, we established that the sulfonylurea tolazamide stimulated rat mesangial cells (MC) to secrete increased amounts of collagen [25, 26]. As in other extrapancreatic tissues in which sulfonylureas amplify glucose uptake, we established that the enhancement of MC glucose uptake occurred coincident with increased cytosol-to-membrane translocation of the glucose transporter, GLUT1 [26, 27]. The augmentation of hexose uptake was associated with an increased secretion of autocrine transforming growth factor beta-1 (TGF-β1) and collagen accumulation. In addition, glibenclamide induced sustained oscillatory Ca^{2+} transients in MC that coincided with heightened cell contractility [28–33]. Further postulation that these biological effects resulted from binding of sulfonylureas to membranous receptors resulted in the initial characterization of several low-affinity MC SUR2. One of these was represented by a unique and dominant 5.5 kb SUR2 transcript, which we identified as mesangial SUR2B (mcSUR2B) [28]. With the prospect that this transcript and possibly others encoded the SUR2 subunit of a mesangial K_{ATP}, we initiated a series of investigations to characterize the components that would comprise this channel. The initial molecular characterization of the mesangial K_{ATP} would thus render greater insight toward our understanding of the cell-signaling events underlying our previously observed functional data regarding sulfonylureas and MC.

METHODS

Animals

Male Munich-Wistar rats, weighing 150 to 200 g, were obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were housed in accordance with good practice guidelines established by AALAC-approved animal care facilities of Henry Ford Hospital. Animals were fed standard Purina rat chow (Ralston Purina Co., St. Louis, MO, USA) and permitted free access to water.

Reagents

Insulin-transferrin-selenium pre-mix was obtained from Becton Dickinson Biosciences (Franklin Lakes, NJ, USA). Dimethyl sulfoxide (DMSO), glibenclamide, antibiotics, Tween-20, and morpholinopropane sulfonic acid-EDTA-sodium acetate (MESA) buffer were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). RPMI 1640 culture medium, fetal calf serum (FCS) and RNA sizing ladders were obtained from Life Technologies (Gaithersburg, MD, USA). QuikHyb solution was purchased from Stratagene (La Jolla, CA, USA). SMART-RACE™ cloning kits and Advantage cDNA PCR enzyme mixes were obtained from Clontech (Palo Alto, CA, USA). TOPO TA cloning kits and pCR2.1/TOPO vectors were purchased from Invitrogen (Carlsbad, CA, USA). RNA STAT-60 reagent was obtained from Tel-Test, Inc. (Friendswood, TX, USA). Poly(A)$^+$ RNA was isolated by magnetic bead separation (Roche Molecular Biochemicals, Indianapolis, IN, USA). [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Bicinchoninic acid protein assay kits were purchased from Pierce Labs (Rockford, IL, USA). Sodium dodecyl sulfate-polyacrylamide gels (10%) were obtained from Bio-Rad (Hercules, CA, USA). Chemiluminescent detection system kits (ECL) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The 4.3 kbp *Eco RI* restriction of rat SUR2 cDNA (pJGAR7) was generously provided by Dr. Charles Burant (University of Michigan, Ann Arbor, MI, USA). Kir6.1/2 primer sets were gifts of Dr. Stephen C. Hebert (Yale Univ., New Haven, CT, USA). Rabbit anti-SUR2 immune serum was kindly donated to our laboratory by Dr. Susumu Seino (Chiba University, Tokyo, Japan).

Tissue culture

The cells used in all studies were primary MC outgrowths of glomerular explants of Munich-Wistar rats or our well characterized cloned rat mesangial cell line $16KC_2$ [26–28]. The latter are spindle-shaped cells that form characteristic hillocks in confluent tissue cultures and stain positively for vimentin, desmin and α-smooth muscle actin, but not for cytokeratin or factor VIII. Cellular contraction occurs following exposure to either angiotensin II or arginine vasopressin. These cells also bear atrial natriuretic peptide (ANP) receptors and the Thy-1 antigen on their surfaces. Except where noted, primary MC or $16KC_2$ were seeded at a density of 5,000 cells per cm^2 and cultured to near-confluence in RPMI 1640 medium, containing 8 mmol/L glucose, antibiotics (penicillin 100 U/mL; streptomycin 100 μg/mL) and 20% serum.

Cloning by rapid amplification of cDNA ends (RACE)

Poly(A)$^+$ RNA was isolated on magnetic beads from primary cell cultures at passage 8. RACE cloning was carried out with the SMART-RACE™ system [7]. Briefly,

Table 1. Gene-specific polymerase chain reaction (PCR) primers

Primer	Sequence
GSP1B	5′-tccttccagggtctgcatctcaccaa-3′
GSP2B	5′-AGGTGACGAACGGATACTTCTCATGG-3′
GSP3B	5′-aggaggagagacgatttgccacaacc-3′
GSP4B	5′-TGACATTCGAATTCCAACAGGTCAGC-3′
SUR013F	5′-CCTGGAGGTTCAGACTCTTGG-3′
SURORF5	5′-GTCGACGTCCTTCAAGATGGACAGAAC ACTGC-3′
SUR227R	5′-gccttaggaagcagtgttctgtccat-3′
KIR61–2F	5′-AGCCACTGACCTTGTCAACC-3′
KIR61–2R	5′-ggagtcatgaattgcacctt-3′
KIR62–5F	5′-CGTCACAAGCATCCACTCCT-3′
KIR62–3R	5′-cacctgcatatgaatggtgg-3′
KIR62ORF5	5′-GGCCAACGGAGCCATGCTGT-3′
KIR62ORF3	5′-ggacaaggaatccggagagatgc-3′
KIR61ORF5	5′-GGAAGATGCTGGCCAGGAAGAGC-3′
KIR61ORF3	5′-ggattctgatgggcactggtttc-3′
SUR2BORF5	5′-GCAGGCGGTTGGAAGGTAGATCA-3′
SUR2B3NT	5′-catgtccgcacgaacgaacgag-3′
SURS4508F	5′-GGCTCTGGGAGGCTCT-3′

Upper case letters denote the forward primer and lower case letters the reverse primer.

1 μg of MC poly(A)$^+$ RNA was used as template in separate reverse transcription reactions that yielded 5′- and 3′-RACE ready single-stranded cDNA libraries. The primary 5′-RACE reactions were carried out with the primers UPM and GSP1B (Table 1) and nested 5′-RACE reactions with primers NUP and GSP3B. Primary 3′-RACE reactions were carried out with the primers UPM and GSP2 and nested 3′-RACE reactions with primers NUP and GSP4B. The 0.6 and 2.5 kbp 5′-RACE and the 3.9 and 4.3 kbp 3′-RACE generated amplicons were gel-purified for direct T-A subcloning into the pCR2.1/TOPO vector. Automated fluorescent DNA sequencing of the mcSUR2B and rSUR2B RACE fragments was performed on both strands by the ABI® Prism system with the Big Dye™ Terminator Sequencing protocol (Applied Biosystems, Foster City, CA, USA). Consensus sequences were analyzed with MacVector 6.0 and Assembly*LIGN* software (Genetics Computer Group, Inc., Madison, WI, USA) [34]. Finally, to establish the presence of full length, contiguous SUR2 coding sequences as predicted from the RACE cloning results, gene-specific primers SUR2BORF5 and SUR2BORF3 from rSUR2B and SURORF5 and SUR2BORF3 (from mcSUR2B) (Table 1) were designed to amplify the full coding sequences of the respective cDNAs. The 5′-RACE ready, single-stranded primary MC cDNA library was used as template in RT-PCR reactions. Nucleotide sequences were verified by bidirectional sequencing.

Northern analysis

Total RNA was isolated from 16KC$_2$ cells, primary MC at passage 8 and from rat kidney, heart and brain in RNA STAT-60 solution [34]. Twenty-microgram aliquots of total RNA were fractionated on 0.4 mol/L formaldehyde gels at 100V. RNA was transferred to charged nylon membranes by capillary action, UV light-cross-linked and hybridized at 68°C in QuikHyb to cDNA probes, generated by random-priming in the presence of [α-^{32}P]dCTP. To evaluate SUR2 gene expression, three cDNA fragments were designed as templates for probe synthesis. The first probe was unique to the full rSUR2B sequence and synthesized from a 1.9 kbp *Sac I* fragment of the 2.5 kbp 5′-RACE product. A second 215 bp probe was generated by polymerase chain reaction (PCR) with primers, SUR013F and SUR227R, unique to the 5′-mcSUR2B sequence. A third probe was designed to detect both mesangial SUR2 transcripts and was a 4.3 kbp *Eco RI* restriction fragment of pJGAR7. Multiple 20-μg aliquots from the same RNA harvest from 16KC$_2$ cells were probed in Northern analysis with the described probes. Changes in SUR2 gene expression in response to glibenclamide were studied in serum-deprived half-confluent cultures, 24 and 48 hours following the addition of 5 μM glibenclamide or vehicle. In these experiments, 20 μg aliquots of RNA were probed with pJGAR7 during Northern analysis [28]. The 1.2 kbp coding sequences of Kir6.1 and Kir6.2 were used as templates for specific probe generation in Northern analysis of RNA derived from primary MC and 16KC$_2$ cells, kidney cortex, and the positive control, heart. Membranes were washed twice at 25°C for 10 minutes in 2× standard sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) and once at 60°C for 20 minutes in 0.1× SSC/0.1% SDS. Membranes were autoradiographed and films examined after 12 to 72 hours at −70°C. These experiments were conducted three times.

Microdissection of glomeruli and RNA isolation for RT-PCR

Male Munich-Wistar rats were sacrificed and microdissection of glomeruli and RNA isolation was performed as described previously [35].

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out on 1 μg of total RNA extracted from rat tissues, 20 ng of MC poly(A)$^+$ RNA, or 5 μL of glomerular RNA isolate with a single tube system. The gene expression of previously described major SUR2 splice variants was explored by RT-PCR in tissues and primary MC [6]. Primers SURS4508F and SUR2B3NT were designed to specifically amplify a 446 bp SUR2B fragment and a 621 bp SUR2A fragment, to fully resolve whether MC expressed the characteristic N-terminal 38A exon of cardiac SUR2A [7, 11]. Primers, SUR013F and SUR227R, were designed to specifically amplify a 215 bp fragment of the unique 5′-mcSUR2B sequence. In separate experiments conducted to detect Kir6.x gene expression by primary MC, we used the

Kir6.1 gene-specific primer pair, KIR61-2F and KIR61-2R, and the Kir6.2 gene-specific primer pair, KIR62-5F and KIR62-3R, which were designed to amplify, respectively, a 402 bp and a 318 bp fragment. Amplicons were visualized on ethidium bromide stained gels. Finally, to obtain templates for radiolabeling in Northern blotting studies, the 1.2 kbp coding sequences of Kir6.1 and Kir6.2 were amplified by RT-PCR of MC poly(A)$^+$ RNA and heart total RNA with sequence-specific primer pairs for Kir6.1, KIR61ORF5 and KIR613ORF3, and for Kir6.2, KIR62ORF5 and KIR62ORF3, respectively [15, 36]. Nucleotide sequences of the amplicons were verified by DNA sequencing. Three independent experiments were conducted.

Immunoblotting

Confluent primary MC and $16KC_2$ monolayers were scraped into an ice-cold buffer (pH 7.4), containing 20 mmol/L HEPES, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 250 mmol/L sucrose, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L pepstatin, and 1 mmol/L leupeptin, then immediately homogenized in a glass dounce [26]. Brain tissue was snap-frozen in liquid nitrogen immediately following sacrifice of the animal and pulverized in a liquid nitrogen-cooled mortar, then homogenized. Tissue debris and nuclei were pelleted out by centrifugation at $1000 \times g$ for 10 minutes three times. Crude membrane preparations were obtained by centrifugation of clarified supernatants at $100{,}000 \times g$ for 60 minutes. Subsequently 20-μg aliquots of total protein from membrane-enriched fractions of MC, $16KC_2$ cells, or brain were separated by electrophoresis on 10% SDS polyacrylamide slab gels. Membranes were blocked in 5% dry milk/0.1% Tween/1× PBS solution for 60 minutes and incubated with anti-SUR2 rabbit antiserum (1:1000 dilution) that reacts with a conserved region of rat SUR2A/B close to the C-terminus. Following incubation with anti-rabbit HRP-conjugated secondary antibody, SUR2 proteins were demonstrated by a chemiluminescent assay [26, 34].

RESULTS

RACE cloning of mesangial SUR2

The nested 5′-RACE reaction generated amplicons of 0.6 kbp and 2.5 kbp and the nested 3′-RACE reaction yielded amplicons of sizes 3.9 kbp and 4.3 kbp. The 3.9 kbp 3′-RACE amplicon was identical to the 4.3 kbp product but was truncated by 400 bp in the 3′ untranslated region (UTR). Alignments of the 2.5 kbp 5′-RACE and 4.3 kbp 3′-RACE contigs spanned the 4.6 kbp rSUR2B cDNA coding region and reconstructed a 6.7 kbp SUR2B sequence. Within the coding sequence, 100% identity with the rSUR2B sequence was established [37]. Further analysis demonstrated a unique 160 bp sequence in the 2.5 kbp 5′-RACE product's 5′-UTR. In addition, the 0.6 kbp 5′-RACE and 4.3 kbp 3′-RACE contigs comprise the novel 4.8 kbp mcSUR2B. The presence of the two separate mesangial SUR2B cDNAs was confirmed by end-to-end PCR with primers specific to each coding region in reactions with cDNA templates from a single-stranded 5′-RACE rat cDNA library.

The 6.7 kbp SUR2B reading frame is 4635 bp and the translated protein of 1535 aa is identical to rSUR2B. The 4.8 kbp mcSUR2B reading frame is 2892 bp and translates a protein of 964 aa. This peptide has a novel 16 aa insert at the beginning of its amino terminus and is succeeded by 948 aa that are identical to rSUR2B in the region spanning exons 13 to 38B. The peptide alignment of the two mesangial SUR2 is displayed in Figure 1. Based on sequence identity between mcSUR2B and rSUR2B, the predicted topology of mcSUR2B as compared to rSUR2B is displayed in Figure 2 and demonstrates that mcSUR2B lacks the initial 12 rSUR2B exons [4]. This deletion renders a model whereby the mcSUR2B N-terminus is intracytoplasmic. The putative mcSUR2B retains the endoplasmic reticulum retention signal RKQ that prevents cell surface expression of incompletely assembled K_{ATP}. It also has two nucleotide binding folds, NBF1 and NBF2, that contain the Walker A and B motifs common to all SUR, and the terminal exon 38B that imparts diazoxide sensitivity to SUR2B-containing K_{ATP} [38]. McSUR2B is predicted to have six transmembrane helices whose peptide sequence is identical to the N-terminal transmembrane domain (TM2) of rSUR2B. The binding sites for the potassium channel openers pinacidil and cromakalim were previously localized to this area in SUR2A and SUR2B [39–42]. The cytoplasmic side of the homologous domain in SUR1 provides a high affinity-binding site for the sulfonylurea agents, tolbutamide and glibenclamide. Compared to SUR1 the TM2 in SUR2 has a significantly lesser affinity for sulfonylureas [43–45].

Mesangial SUR2 gene expression

These studies were performed to determine whether the smaller SUR2 4.8 kbp cDNA obtained by RACE cloning represents the dominant 5.5 kbp transcript, which we previously detected by Northern analysis with a SUR2 probe (pJGAR7, 28). We generated a 1.9 kbp *Sac I* fragment from the 2.5 kbp 5′-RACE product, unique to the rSUR2B cDNA. Predictably, this fragment hybridized only to the mesangial SUR2 transcript at 7.5 kbp. Using PCR, a 215 bp probe was generated corresponding to the unique 5′-sequence of mcSUR2B. This probe preferentially hybridized to the novel 5.5 kb SUR2 message, confirming that the 4.8 kbp cDNA represented the 5.5 kb SUR2 mesangial transcript. Finally, the 4.3 kbp *Eco RI* fragment of pJGAR7 that contains sequence common to both the 4.8 and 6.7 kbp cDNAs predictably

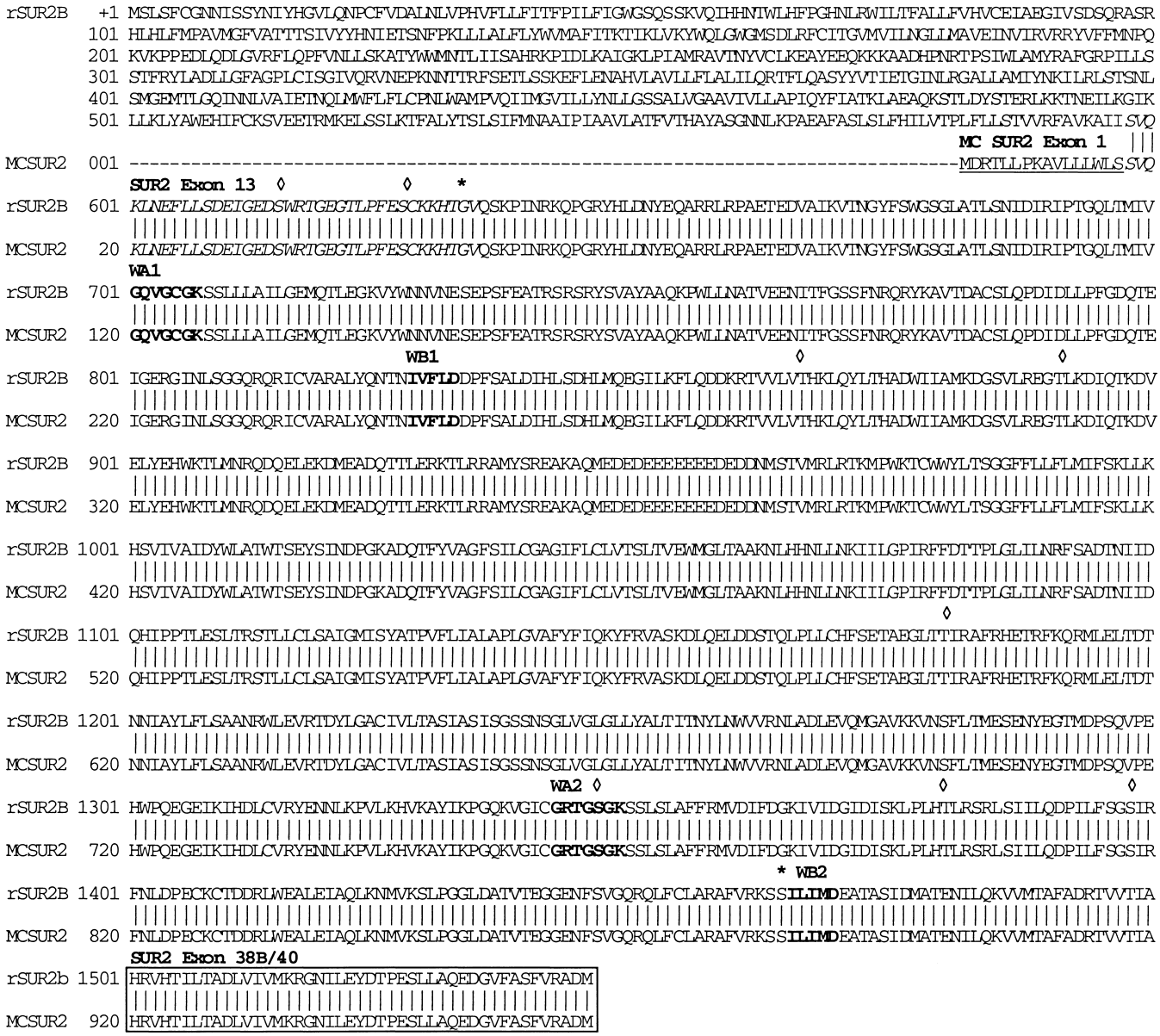

Fig. 1. Primary sequence of rat mesangial type 2 sulfonylurea receptors (SUR2). The respective 4635 bp and 2892 bp open reading frames of rat SUR2B (rSUR2B) and mesangial SUR2B (mcSUR2B) cDNAs were translated, and the putative amino acid sequences are displayed. Vertical bar (|) denotes sequence identity. The underscore (___) defines the sequence unique to mcSUR2. Italics show the amino acids that correspond to exon 13 of rSUR2B that begins region of identity between rSUR2B and mcSUR2B. Bold type illustrates the Walker A and B ATP-binding motifs. Asterisks (*) denote the protein kinase A-dependent phosphorylation sites, open diamonds (◇) are the protein kinase C-dependent phosphorylation sites, and the boxed region (□) shows the terminal SUR2B-specific, alternatively spliced exon.

hybridized to SUR2 transcripts at 5.5 and 7.5 kb (Fig. 3). Finally, to establish whether the novel mcSUR2B splice variant was expressed in vivo, RT-PCR was performed on RNA template isolated from microdissected rat glomeruli. Using a primer pair that amplified the unique 5′-sequence of mcSUR2B, we confirmed the gene expression of the mcSUR2B splice variant in rat glomeruli. In similar RT-PCR reactions, a large amount of specific product was observed using RNA template from $16KC_2$ or MC (Fig. 4B).

A major difference among SUR2 splice variants is the alternative usage of either the terminal 38A exon that characterizes SUR2A or its 38B counterpart that establishes SUR2B. With gene-specific primers, 38A/B exon usage was determined in MC. A 446 bp amplicon was amplified in MC, signifying alternative splicing of only the 38B exon (Fig. 4A). In parallel studies of heart (positive control) and kidney, 446 and 621 bp amplicons were amplified in heart, but only a 446 bp amplicon was detected in kidney. Collectively, these data support SUR2A

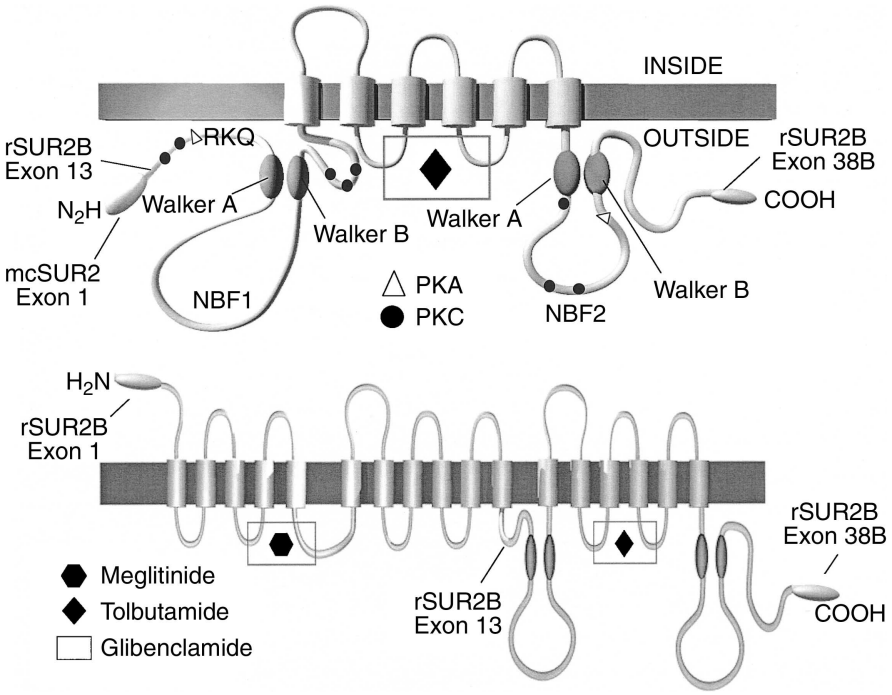

Fig. 2. Putative topology of the mesangial SUR2 protein and rat SUR2B. (*Top*) The rat mesangial SUR2B (mcSUR2B) protein is comprised of a single transmembrane spanning domain (TM) with 6 helices that are flanked by two cytoplasmic loops. Each loop contains nucleotide-binding folds (NBF) with Walker A and B motifs that bind and hydrolyze ATP. The *N*-terminus is predicted as intracytoplasmic, distinct from other SUR. The putative endoplasmic reticulum retention signal RKQ is shown. Protein kinase A (△)- and protein kinase C (●)-dependent phosphorylation sites and the proposed binding sites of various sulfonylurea compounds are indicated. (*Bottom*) Rat SUR2B topology.

and SUR2B isoform expression in heart, but only SUR2B in kidney [6].

Because our previous observations implied that sulfonylureas, possibly through SUR2B, regulate MC contractility and extracellular matrix metabolism, investigations were carried out to determine whether glibenclamide regulated the mesangial expression of the *SUR2B* gene. In two independent studies, glibenclamide (5 μM) did not influence SUR2B expression in serum-deprived MC after 48 hours of drug exposure (Fig. 5). Longer incubation times were not evaluated. Because the 5.5 kb SUR2B mesangial transcript became more evident during growth of cells in culture (data not shown) and because Kir6.1 transcripts increased during embryonic development, we sought to determine whether serum regulated SUR2B expression. Interestingly, the level of the major 5.5 kbp transcript became undetectable following a 24-hour period of serum deprivation and this change persisted for at least 48 hours. In contrast, serum-deprivation did not alter expression of the 7.5 kb transcript (Fig. 5).

Mesangial SUR2 protein detection

Using polyclonal anti-SUR2 antibody, multiple bands were detected from M_r 50 to 170 kD in MC (Fig. 6). By contrast, in the positive control brain tissue only a 170 kD band was visualized. In membrane-enriched preparations of primary MC and $16KC_2$ cells, a dominant band was detected at 108 kD, consistent with the predicted size of the translated mcSUR2B peptide. Lastly, lesser signal intensity was evident at ~170 kD.

Mesangial Kir6.1 and 6.2 gene expression

To establish mesangial Kir6.1/2 gene expression, RT-PCR was performed with gene-specific primers. In MC, a 402 bp fragment corresponding to Kir6.1 was robustly amplified, whereas the 318 bp fragment of Kir6.2 was minimally evident (Fig. 7A). As the *Kir6.2* gene is intronless [11], the possibility existed that amplification of contaminating genomic DNA had contributed to these results. However, PCR, in the absence of reverse transcription generated no Kir6.2 products negating this possibility. Lastly, in Northern analysis, transcripts of Kir6.1 were strongly detected at 1.7 and 2.7 kb in primary MC, kidney cortex, whole kidney, and heart. The Kir6.2 transcript of 4.1 kb was only identified in the positive control heart (Fig. 7B, C).

DISCUSSION

Data from Metzger and colleagues has previously implied the presence of glomerular SUR [46, 47]. This group demonstrated both low- and high-affinity glibenclamide binding in metabolically active preparations of rat glomeruli. However, the cell type(s) responsible for the binding were not unequivocally described and the presence of contaminating vasculature in their preparations may have accounted for the drug binding. This is plausible because the existence of glomerular SUR, as a component of afferent arteriolar $K_{AT}P$, has been established [48]. Moreover, sulfonylurea drug binding to glomerular K_{ATP}-bearing renin-producing cells also may have accounted for their results [49].

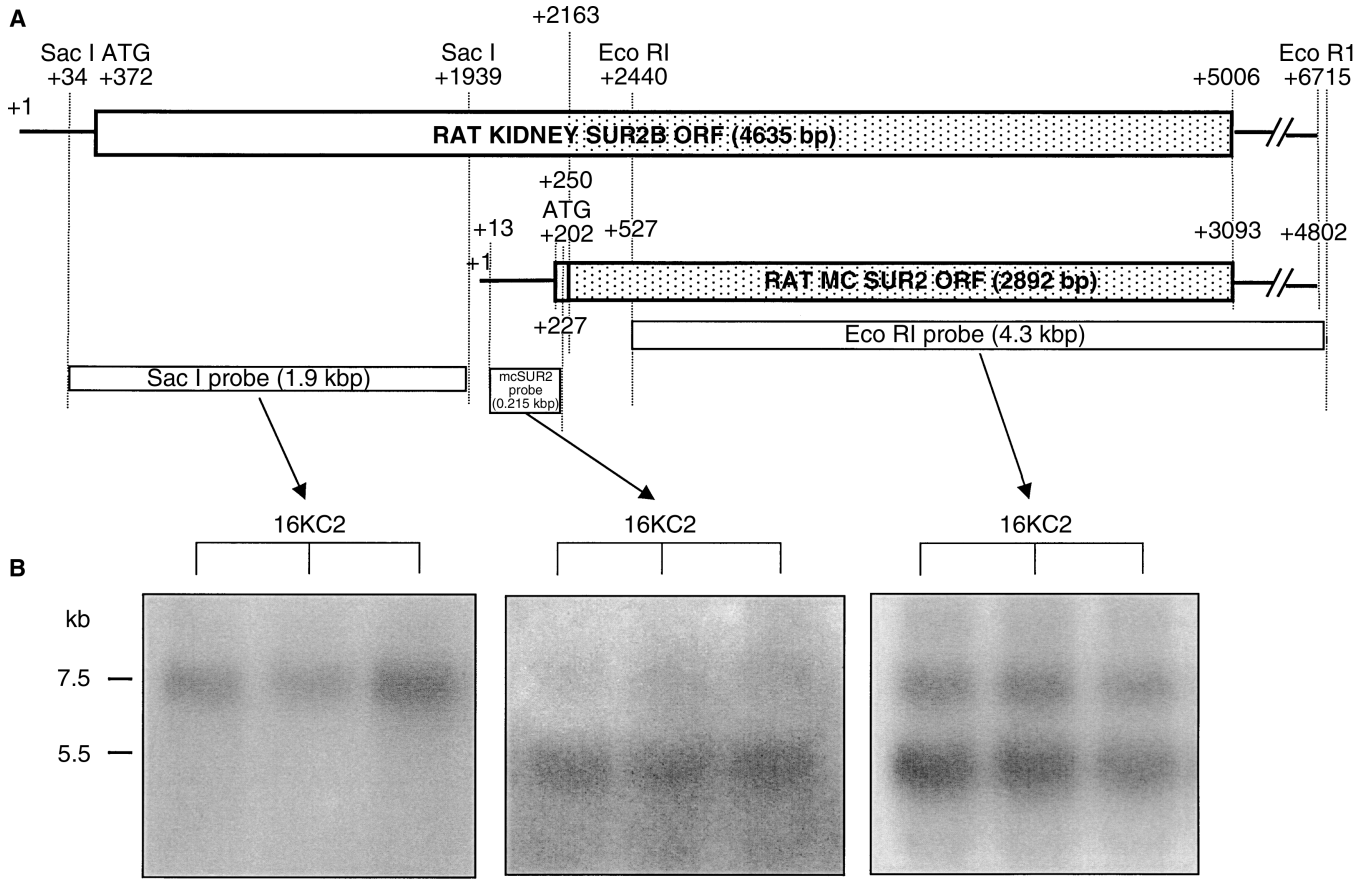

Fig. 3. Mesangial SUR2 gene expression. Differential identification of rat mesangial cell transcripts for SUR2B was carried out with gene-specific probes in northern analysis as described in the **Methods** section. (*A*) Rat kidney SUR2B (rSUR2B) and mesangial SUR2B (mcSUR2B) cDNAs are shown. Stippled portions correspond to regions of identity. *Sac I* sites (+34 to +1939 bp) define an rSUR2B-specific probe. *Eco RI* sites (+2440 to +6715 bp) define a probe that detects rSUR2B and mcSUR2B transcripts (pJGAR7). A 215 bp 5′-terminal fragment (+13 to +227 bp) defines the mcSUR2B-specific probe. (*B*) The *Sac I* probe only detects the 7.5 kb SUR2B transcript. The mcSUR2B-specific probe strongly hybridizes to the 5.5 kb transcript. The pJGAR7 probe hybridizes to transcripts at 5.5 and 7.5 kb.

Our data provide convincing evidence for the existence of at least two mesangial SUR2B, which likely heterodimerize with Kir6.1 to form mesangial K_{ATP}. Two rat mesangial SUR2B cDNAs were RACE-cloned, and the cDNAs corresponding to the two most abundantly expressed mesangial SUR2 transcripts of 5.5 and 7.5 kb characterized by Asano et al [28]. To date, the longest SUR2B clone that has been isolated is the 6.6 kb rSUR2B cDNA obtained from a rat kidney library [37]. The coding region of our 6.7 kb SUR2B cDNA was 4635 bp and essentially identical to rSUR2B, except for several nucleotide differences that may represent species-specific variations and that do not affect the primary sequence. In addition, the 5′-UTR of the 6.7 kb clone contains a novel 160 bp sequence, which we presume resulted from cell-specific alternative splicing.

Despite the 0.7 kbp difference in length compared to the 5.5 kb mesangial SUR2B transcript, we ascertained that our 4.8 kbp cDNA contains the same open reading frame as the larger message. Perhaps the foreshortening of this clone may be attributable to an inability of our RACE-based system to negotiate complex topology in the 5′-UTR. Nevertheless, the sequence of mcSUR2B, the smaller mesangial SUR2B that corresponds to the 5.5 kb transcript, clearly identifies it as a member of the SUR2 family. From aa 17 to 964, mcSUR2B shares identity with the region of rSUR2B that spans exons 13 to 38B. However, mcSUR2B is truncated at its N-terminus. This gene has a deletion of the 12 proximal N-terminal rSUR2B exons and a unique 16 aa insert at its amino terminus, discriminating it from other SUR2 (Figs. 2 and 3). Our topological analysis of mcSUR2B reveals a single, 6-helix transmembrane spanning domain. By contrast, rSUR2B has three transmembrane domains (Fig. 3) [4, 37]. Additionally, the mcSUR2B N-terminus is predicted to be intracytosolic, as opposed to rSUR2B and other SUR2 wherein the N-terminus resides extracellularly [4]. Lastly, mcSUR2B retains the transmembrane domain corresponding to TM2 of rSUR2B. The cytosolic side of this domain binds the

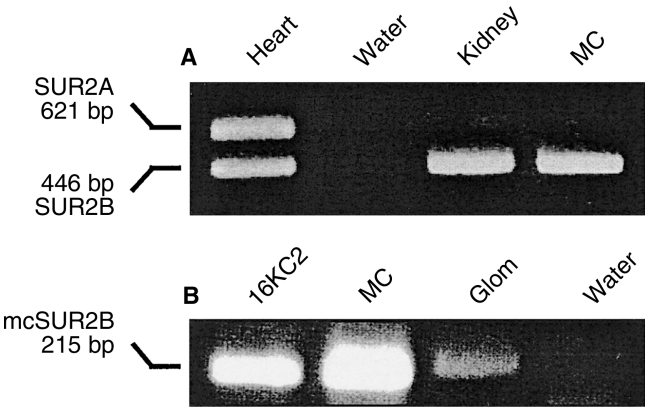

Fig. 4. Expression of SUR2 splice variants in rat glomerulus and mesangial cells (MC). RT-PCR reactions were carried out in rat heart, kidney, primary MC, and microdissected glomeruli, with gene-specific primers that define expression of mcSUR2B, SUR2A, or SUR2B as described in the **Methods** section. Gene products were visualized on ethidium bromide stained gels. (*A*) SUR2A and SUR2B are detected in heart. In kidney and MC, only SUR2B is expressed. No gene product is seen in the negative control (water). (*B*) The unique 215 bp 5′-terminal fragment of mcSUR2B is detected in $16KC_2$ and MC and microdissected glomeruli. No gene product is detected in the negative control (water).

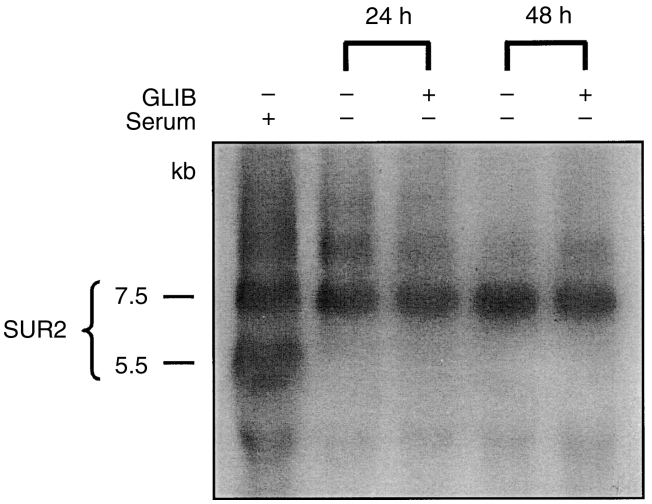

Fig. 5. Serum deprivation induces differential gene expression of SUR2B. Gene regulation of SUR2B by glibenclamide and serum was examined. Subconfluent mesangial cell cultures were maintained in 20% serum or serum-deprived and exposed to glibenclamide (5 μM) for times shown. In Northern analysis, a probe that detects both the 7.5 kb rat SUR2B and the 5.5 kb mesangial SUR2B transcript was used (pJGAR7). Serum-stimulated cells express 5.5 and 7.5 kb transcripts. After a 24-hour serum deprivation, the 5.5 kb mesangial-specific transcript is undetectable and this down-regulation persists for at least 48 hours. Exposure to glibenclamide did not influence SUR2 gene expression.

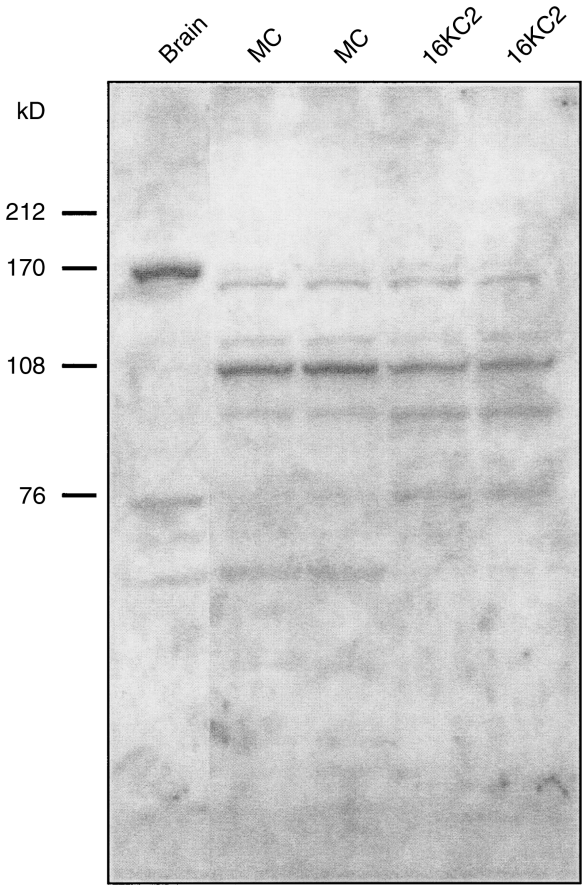

Fig. 6. Detection of mesangial SUR2 proteins. Immunoblotting of SUR2 proteins was carried out on membrane-enriched fractions of rat brain, mesangial cells (MC) and $16KC_2$ cells. The rabbit polyclonal SUR2 antiserum detects common epitopes of SUR2A and SUR2B near their C-termini as described in the **Methods** section. In the positive control tissue brain, the rat SUR2B is strongly detected at 170 kD. In MC and $16KC_2$ cells, the mesangial SUR2B is detected at M_r 108 kD, while the rat SUR2B is less strongly detected at M_r 170 kD.

potassium channel opener agents, pinacidil and cromakalim, with high affinity [39–42], and also contains a low-affinity sulfonylurea binding site [43–45].

In immunoblotting experiments, the apparent molecular mass of 108 kD of the dominant band agrees with the size predicted from translation of the 2892 bp open reading frame of the 4.8 kbp mcSUR2B clone. The less strongly evident 170 kD band most likely characterizes expression of the 6.7 kbp rSUR2B cDNA. Previous immunoblotting studies of protein isolates from cell lines transformed by SUR2A or SUR2B cDNA clearly demonstrated proteins with an apparent mass of 170 kD [50]. Interestingly, these full-length proteins were not detected from cardiac sarcolemma [51] or whole mouse kidney crude membrane preparations [52], although these tissues express full size SUR2A and SUR2B, respectively [7, 20, 37, 52]. Presumably, immunodetection of full length SUR2 in MC (and murine cardiac sarcolemma and whole kidney crude membrane preparations) is difficult. On the other hand, the 108 kD band corresponding to the abundant 5.5 kb mcSUR2B transcript is convincingly demonstrated and the relative intensities

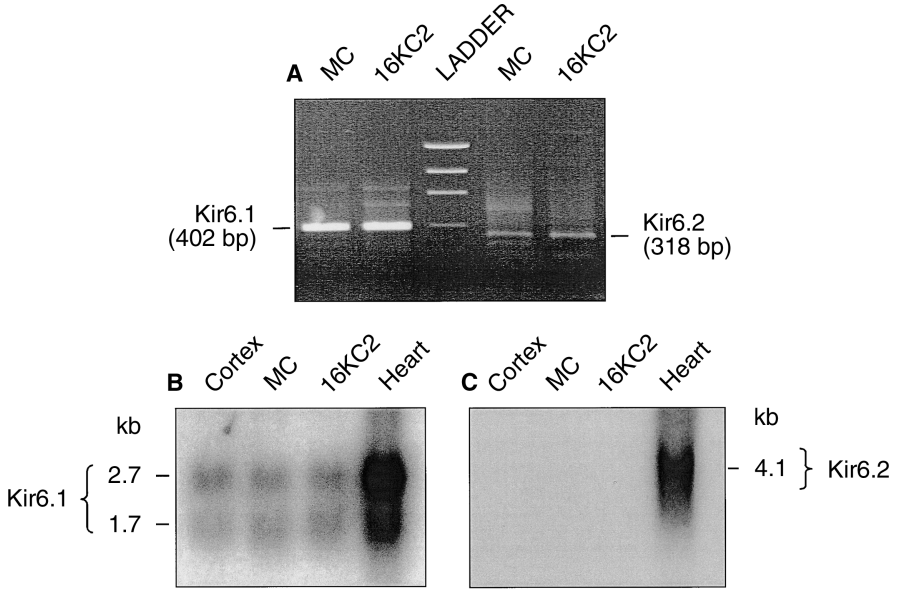

Fig. 7. Mesangial Kir6.x gene expression. RT-PCR reactions with gene-specific primers of Kir6.1 and Kir6.2 were carried out on $16KC_2$ cells and mesangial cells (MC). Reaction products were visualized on an ethidium bromide-stained gel. (*A*) Strong Kir6.1 gene expression is shown for MC and $16KC_2$ cells, while only minimal Kir6.2 gene expression is detected. (*B*) Kir6.1 cDNA was used as probe in northern analysis. Hybridization to transcripts at sizes shown is seen in renal cortex, MC, $16KC_2$ cells, and the positive control, heart. (*C*) Parallel studies with Kir6.2 cDNA only detected a transcript in the heart.

of the 170 kD and 108 kD signals on immunoblotting roughly correspond to their hybridization signal intensities previously determined by Northern blotting [28].

The human *SUR2* gene spans more than 100 kbp and contains no less than 39 exons [11]. Multiple tissue-specific splice variants of *SUR2* have been documented in Northern assays and by RT-PCR, and these variations may contribute to the functional diversity of K_{ATP} [11]. The cDNAs characterized by these studies shared extensive homology with mcSUR2B; however, the 5.5 kb transcript of mcSUR2B was not detected previously in cells or tissues evaluated, suggesting that MC carry out a unique alternative splicing event. The two splice variants, cardiac SUR2A and the vascular smooth muscle cell (VSMC) SUR2B, differ in their expression of the 38A/B C-terminal exon. A deletion of exon 38A, with preferential expression of exon 38B in SUR2B, renders VSMC K_{ATP} sensitive to the K^+ channel opener and vasodilator, diazoxide [41, 42]. In contrast, cardiac K_{ATP}, a tetradimer of SUR2A and KIR6.2, is diazoxide-insensitive. Our results indicate the exclusive usage of exon 38B by MC and kidney, consonant with the report of Beesley and colleagues who characterized the expression of murine kidney SUR2 [52].

Thus far, regulation of the constitutively expressed *SUR2* gene has not been defined. However, prolonged exposure of pancreatic β cells to glibenclamide has been shown to increase SUR1 protein expression and to affects its translocation from the cytosol to membrane [53]. We examined the effects of cyclical mechanical stretch (data not shown) and glibenclamide on mesangial SUR2 gene expression because sulfonylureas are typically utilized as therapy in diabetic individuals whose glomeruli are exposed to the pernicious effects of glomerular hypertension and the concomitant distensile forces. However, there was no alteration of MC SUR2 gene expression by either stimulus, even after 48 hours of exposure to glibenclamide and stretch. However, we did document that serum deprivation of 24 to 48 hours potently downregulated gene expression of the 5.5 kb mcSUR2B transcript, while transcript levels of the 7.5 kb message remained unchanged. The physiological consequence of this differential expression is unknown.

In VSMC, SUR2B partners with Kir6.1 to form K_{ATP}, and more rarely, Kir6.2 may represent the ion pore-forming subunit. Analogously, for MC to furnish an ion pore channel to mesangial K_{ATP}, they must also express Kir6.x. In MC, we established robust gene expression of Kir6.1, and minimal Kir6.2 expression in RT-PCR and Northern blotting studies. Consequently, our data favor the assembly of two mesangial K_{ATP}, heterodimers of $(rSUR2B:Kir6.1)_4$ or $(mcSUR2B:Kir6.1)_4$ [22]. Several investigations have established that the various SUR2 exhibit differential electrophysiological characteristics [54, 55]. Based on a recent study of Kir6.2 knockout mice, Kir6.1 represents the functional pore-forming subunit of VSMC K_{ATP} [56]. With analogy to VSMC K_{ATP}, one of the mesangial K_{ATP} $(rSUR2B:Kir6.1)_4$ would be predicted to be activated by the potassium channel openers, pinacidil, nicorandil and diazoxide [39–42] and inhibited by sulfonylureas [43–45]. In addition, this particular K_{ATP} may be nucleoside diphosphate-regulated rather than ATP-inhibited [22, 57, 58]. Finally, the second mesangial would be assembled as $(mcSUR2B:Kir6.1)_4$ and could manifest an electrophysiologic profile different than $(rSUR2B:Kir6.1)_4$.

Given the ubiquitous expression of K_{ATP} and their regulation by sulfonylureas, the question arises as to

whether endogenous ligands for these channels exist [59]. At least for pancreatic β cell K_{ATP} $(SUR1:Kir6.2)_4$, there is one such "endogenous sulfonylurea:" α-endosulfine. This 121 aa polypeptide binds SUR1 with high affinity and stimulates insulin release through K_{ATP} inhibition [60–62]. We have recently demonstrated that α-endosulfine gene (*ENSA*) is expressed in rat MC and glomeruli (abstract, *J Am Soc Nephrol* 11:448A, 2000). We speculate that autocrine endosulfine mediates sulfonylurea-type effects via SUR2B. The existence of such an autocrine, and possibly paracrine, system could have broad implications for diabetic glomerulopathy with its attendant pathophysiologic alterations of mesangial cell contractility and matrix metabolism.

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Reprint requests to Jerry Yee, M.D., Division of Nephrology and Hypertension, Henry Ford Hospital, 2799 West Grand Boulevard, CFP-514, Detroit, Michigan 48202, USA.
E-mail: jyee1@hfhs.org

APPENDIX

Abbreviations used in this article are: ABC, ATP-binding cassette; ANP, atrial natriuretic peptide; ATP, adenosine 5′-triphosphate; CFTR, cystic fibrosis transmembrane regulator; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; FCS, fetal calf serum; K_{ATP}, ATP-sensitive K^+ channel; Kir6.1, inwardly-rectifying K^+ channel 6.1; MC, mesangial cell; MESA, morpholinopropane sulfonic acid-EDTA-sodium acetate; MRP, multidrug resistance protein; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; SUR, sulfonylurea receptor; SUR2B, type 2B sulfonylurea receptor; UTR, untranslated region; VSMC, vascular smooth muscle cells.

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