Rat homolog of sulfonylurea receptor 2B determines glibenclamide sensitivity of ROMK2 in Xenopus laevis oocyte

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Tanemoto, Masayuki, Carlos G. Vanoye, Ke Dong, Richard Welch, Takaaki Abe, Steven C. Hebert, and Jason Z. Xu. —Rat homolog of sulfonylurea receptor 2B (denoted rSUR2B) reconstituted an inwardly rectifying, ATP-sensitive K+ channel that was inhibited by glibenclamide (2, 15–17). Here we report the isolation of a rat homolog of mouse SUR2B (denoted rSUR2B) from a rat kidney cDNA library. The rSUR2B sequence contains a 4,635-bp open reading frame that encodes a 1,545-amino acid polypeptide, showing 67% shared identity with SUR1 (a pancreatic β-cell isoform) and 98% with both SUR2A (a brain isoform) and SUR2B (a vascular smooth muscle isoform). Consistent with the predicted structures of other members of the ATP-binding cassette (ABC) superfamily, the sequence of rSUR2B contains 17 putative membrane-spanning segments. Also, predicted Walker A and B consensus binding motifs, present in other ABC members, are conserved in the rSUR2B sequence. RT-PCR revealed that rSUR2B is widely expressed in various rat tissues including brain, colon, heart, kidney, liver, skeletal muscle, and spleen. The intrarenal distribution of the rSUR2B transcript was investigated using RT-PCR and Southern blot of microdissected tubules. The rSUR2B transcript was detected in proximal tubule, cortical thick ascending limb, distal convoluted tubule, cortical collecting duct, and outer medullary collecting duct, but not medullary thick ascending limb. This distal distribution overlaps with that of ROMK.

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Kir2.1, or Kir3.4 in X. laevis oocytes failed to confer sulfonylurea sensitivity to these K⁺ channels (2), it appears that the SUR1 interacts only with the Kir6.x subfamily of Kir channels. However, the recent observations that another member of the ABC family, the cystic fibrosis transmembrane conductance regulator (CFTR), can confer glibenclamide sensitivity to ROMK2 in X. laevis oocytes (24, 25, 32) suggests that ROMK may assemble the renal epithelial K⁺ATP channel with other SUR isoform(s) or ABC family members.

In the present study, we report cloning and localization of a rat homolog of the mouse SUR2B (denoted rSUR2B). RT-PCR and Southern blot analyses showed that rSUR2B was expressed in various tissues and widely expressed in rat kidney nephron segments. Coexpression of rSUR2B with ROMK2 in X. laevis oocytes resulted in a glibenclamide-sensitive K⁺ channel, implicating SUR2B in forming and regulating the renal epithelial K⁺ATP channel.

METHODS

cDNA cloning a sulfonylurea receptor from rat kidney. Total RNA (10 µg) from rat kidney was reverse transcribed with an oligo(dT) primer and Moloney murine leukemia virus RT (GIBCO). The resulting cDNA was amplified in the PCR using several pairs of oligonucleotide primers designed according to the DNA sequence of rat SUR2A (16): sense primer, R2As: 5′-CGTGGCCATCGACTACTGGC-3′; alignment position 3011–3031 in rat SUR2A; antisense primer, R2Aa: 5′-GACAGCAGGAAGAGCGGTG-3′; alignment position 1738–1757 in rat SUR2A. The PCR fragments were subcloned into BlueScript vector (Stratagene) and sequenced using the cycle sequencing method (Perkin-Elmer). The PCR fragment homologous to SUR2A was random prime-labeled using [α-32P]dATP (3,000 Ci/mmol, Amersham) and used as a probe to screen a rat kidney cDNA library. Six distinct clones were grouped by PCR and restriction mapping; one representative clone was further sequenced by cycle sequencing. The full length of rSUR2B was subcloned into the pGEMHE vector, which contains a T7 RNA polymerase promoter for eukaryotic in vitro translation, and a Xenopus β-globin gene for functional expression (21).

Tissue distribution of rSUR2B expression. Expression of rSUR2B in renal tubules was determined by RT-PCR and Southern blotting. Young pathogen-free male Sprague-Dawley rats (80–100 g) were anesthetized, and kidneys were perfused initially with 10 ml PBS and then with 10 ml digestion solution [DMEM (GIBCO) containing 0.5 mg/ml collagenase (type I, Sigma), 0.5 mg/ml pronase E (Sigma), and 0.1% antifoam B (Sigma)]. After death of the rat, the perfused kidney was removed, and individual nephron segment microdissection was performed as described previously (7, 20, 38). Average length of dissected tubules was 0.5–1 mm, and three tubules were combined as one sample. Dissected tubules were directly reverse transcribed and amplified by the PCR using primers 2A-1 and 2A-2. The PCR products were displayed on a Southern blot and visualized by hybridization to a 32P-labeled cDNA probe of the full length of rSUR2B coding sequence. Specific hybridization of the rSUR2B probe was quantified by the intensity of the β-actin ethidium bromide-stained bands. Each PCR reaction also included a pair of

Fig. 1. Amino acid sequence comparison of sulfonylurea receptors mSUR2B and rSUR2B from mouse or rat, respectively. The amino acid residues of mSUR2B that differ from those of rSUR2B are shown below rSUR2B (bold). Walker A and B consensus sequences are boxed. Overall amino acid identity between mSUR2B and rSUR2B is 98.7%.
-actin primers that span one intron to control for genomic DNA contamination.

Two-electrode voltage clamp. \( \text{Ba}^{2+} \)-sensitive \( K^+ \) currents in \( X. \text{laevis} \) oocytes injected with cRNA from each of the three ROMK splice variants (ROMK1, -2, and -3; 5 ng), or with rSUR2B (50 ng), or coinjected with rSUR2B and each of the ROMK splice variants (5 ng ROMK, 50 ng rSUR2B; molar ratio = 1 ROMK: 3 rSUR2B) were functionally examined by two-electrode voltage clamping as described (7). Electrophysiological recordings were performed 96 h after RNA injection at 22 ± 2°C by two-electrode voltage clamp at a holding potential of −80 mV (Axoclamp 2A, Axon Instruments). The bath solution was as follows (in mM): 96 NaCl, 1 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), and 5 HEPES (pH 7.4), with 5 mM \( \text{Ba}^{2+} \) or various concentrations (0.1, 0.2, and 0.4 mM) of glibenclamide. The average resting potential was −100 mV (range −70 to −104 mV) at an external \( K^+ \) concentration of 1 mM. The oocyte holding potential was −90 mV. The oocytes were pulsed over the range of −160 to −40 mV every 20 mV for 50 ms. Currents ranged from 0.06 to 0.20 µA at −140 mV with 1 mM external \( K^+ \). As indicated in our previous publication (24), glibenclamide sensitivity is best observed with low external \( K^+ \). Glibenclamide was diluted from a 1,000× stock solution dissolved in DMSO (Sigma). Equal amounts of DMSO were added to the control bath solutions. The EC\(_{50}\) value for glibenclamide block was calculated from the one-site nonlinear regression model performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

In vitro translation of ROMK2 and rSUR2B and immunoprecipitation. Hemagglutinin (HA)-tagged ROMK1, HA-tagged ROMK2 (39) and wild-type rSUR2B cDNA were translated in vitro either separately or together using TNT-coupled reticulocyte lysate system and \(^{35}\)S)methionine in the presence or absence of canine microsomal membranes as per the manufacturer's instruction (Promega). Reaction mixtures were incubated at 30°C for 120 min after addition of \(^{35}\)S)methionine. Immunoprecipitation was performed according to Xu et al. (39) using anti-HA monoclonal antibody (clone 12CA5, Boehringer Mannheim). Protein products were resolved by 8% SDS-PAGE, and the \(^{35}\)S)methionine-labeled ROMK proteins were visualized by autoradiography.

RESULTS
Identification of rSUR2B in rat kidney. A PCR fragment, which showed 99% homology to rat SUR2A, was used to screen a rat kidney cDNA library. We obtained six positive clones after screening \( 1.3 \times 10^5 \) plaques. One of these clones, named rSUR2B (rat SUR2B), was further analyzed by sequencing, which revealed a single open reading frame of 4,635 bp encoding a 1,545-amino acid polypeptide. The rSUR2B polypeptide has been proposed to form 17 membrane-spanning domains based on multisequence alignments of SUR and the multidrug resistance-associated protein subfamily (1, 35). rSUR2B contains two potential nucleotide binding folds with Walker A and B consensus motifs (Fig. 1), like those observed in other cloned SUR isoforms. Also, potential N-linked glycosylation sites, protein kinase (PK) A- and PKC-dependent phosphorylation sites, present in other SUR isoforms are conserved in the rSUR2B sequence. Alignment of the resulting amino acid sequence revealed that rSUR2B...
shared 67% identity with SUR1 (2) and 98% with SUR2A and SUR2B (17, 19). As shown in Fig. 1, 18 amino acid residues of rSUR2B were divergent from those of mouse SUR2B (mSUR2B) (His17, Gln96, Met162, Pro328, Lys329, Thr333, Arg334, Phe335, Ser336, Thr618, Asn651, Val666, Ser771, Asp960, Asp1022, Ile1133, and Phe1281). In addition, Phe336 in mSUR2B was absent in rSUR2B.

rSUR2B transcript expression in tissues. Insight into the tissue distribution of rSUR2B transcripts was obtained by surveying the expression of rSUR2B in rat tissues using RT-PCR. As shown in Fig. 2, the amplicon derived from rSUR2B primers (211 bp) was most abundant in brain, heart, liver, spleen, kidney, and colon, with lesser amounts being detected in skeletal muscle. This pattern of transcript expression is consistent with the distribution of SUR2B in mouse tissue (18). SUR2A (387 bp) was detected in heart and skeletal muscles but was absent in the kidney. The kidney tubule distribution of rSUR2B is shown in Fig. 3. The products derived from the RT-PCR of dissected tubules were displayed on a Southern blot following hybridization with a 32P-labeled rSUR2B-specific probe. The amplicon showed a discrete distribution, being very abundant in proximal tubule (proximal convoluted tubule [PCT], proximal straight tubule [PST]), cortical thick ascending limb (CTAL), and outer medullary collecting duct (OMCD) segments. Lesser intensity was seen in the distal convoluted tubule and connecting tubule segments (Fig. 3). The similarity of the kidney distributions of ROMK2 (38) and rSUR2B is consistent with the possibility that renal epithelial secretory KATP channels in distal nephron segments may be comprised of ROMK2 and rSUR2B.

Heterologous expression of rSUR2B and each isoform of ROMK in X. laevis oocytes. To assess whether the cloned rSUR2B forms glibenclamide-sensitive K+ channels with ROMK, we evaluated Ba2+-sensitive K+ currents in X. laevis oocytes coinjected with cRNA transcribed from cDNA of rSUR2B and three ROMK splice isoforms (ROMK1, -2, and -3). Preliminary studies demonstrated that a molar ratio of 1:3 (coinjection of 5 ng ROMK2 cRNA and 50 ng rSUR2B cRNA in oocytes) was best for studying the interaction of ROMK and rSUR2B. Thus this cRNA molar ratio was used for the functional assays in this study. Figure 4, A and B,
shows the results of these experiments in which rSUR2B was coinjected with each of three ROMK splice variants. The coinjected oocytes exhibited significant Ba\(^{2+}\)-sensitive K\(^+\) currents (0.5–1 µA; external K\(^+\) = 1 mM; Fig. 4A). The inhibitory effect of 0.2 mM glibenclamide on Ba\(^{2+}\)-sensitive K\(^+\) currents, however, was limited to ROMK2; the whole cell K\(^+\) currents with ROMK1 and -3 were unaffected by glibenclamide (Fig. 4, A and B). There was no apparent voltage dependence of the glibenclamide block. We next assessed the concentration dependence of glibenclamide-mediated inhibition Ba\(^{2+}\)-sensitive K\(^+\) currents in ROMK2 and rSUR2B coinjected oocytes. As shown in Fig. 5, K\(^+\) currents compared with controls without glibenclamide (I/I_o) were reduced to 0.73 ± 0.095 (n = 8), 0.54 ± 0.064 (n = 15), and 0.38 ± 0.044 (n = 7) of control with exposure to 0.1, 0.2, and 0.4 mM glibenclamide, respectively. The EC\(_{50}\) for glibenclamide-mediated inhibition of K\(^+\) currents in ROMK2 + rSUR2B coinjected oocytes was 185 µM. Glibenclamide had no significant effect on whole cell K\(^+\) currents in oocytes injected with ROMK2 alone (I/I_o = 0.96 ± 0.08 with 0.2 mM glibenclamide).

Coimmunoprecipitation of ROMK and rSUR2B. A direct interaction between SUR proteins and Kir6 channels is required to form K\(_{ATP}\) channels in neuronal tissues, and this association provides for glibenclamide sensitivity via the SUR subunit (15, 16). To assess whether a direct interaction between ROMK and rSUR2B is needed for the glibenclamide sensitivity of K\(^+\) currents in oocytes, we used an in vitro translation assay to examine the ability of the two proteins to associate. Because our anti-ROMK antibody (38) is weak at immunoprecipitation, influenza virus HA-tagged ROMK constructs (ROMK1-HA and ROMK2-HA) were utilized for this study. We had previously shown that these constructs formed functional channels (39). In addition, coexpression of ROMK2-HA and rSUR2B in oocytes yielded a similar sensitivity to glibenclamide [I/I_o = 0.55 ± 0.06 (n = 5) with 0.2 mM glibenclamide] as that for the untagged ROMK2 construct (Fig. 4).

Figure 6 shows the results of these in vitro translation assay experiments performed in the presence of microsomes. Similar results were obtained in the absence of microsomal membranes (data not shown). In vitro translation of ROMK2-HA (Fig. 6, lane 2) or rSUR2B (Fig. 6, lane 3) yielded the expected 43-kDa (38) and 174-kDa (molecular mass is predicted by amino acid sequence) core proteins, respectively. No significant bands were seen when either ROMK2 or rSUR2B was absent (H\(_2\)O control; Fig. 6, lane 1). Fainter bands representing glycosylation products of
these two proteins are visible just above each of the dense-core bands. With cotranslation of ROMK2-HA and rSUR2B, both the 43- and 174-kDa bands are detected (Fig. 6, lane 4). When ROMK2-HA and rSUR2B were translated separately, the anti-HA antibody precipitated only ROMK2-HA (Fig. 6, lane 5) but not wild-type rSUR2B (Fig. 6, lane 6). In contrast, when ROMK2-HA and rSUR2B are cotranslated, the anti-HA antibody precipitated a complex of ROMK2-HA and rSUR2B (Fig. 6, lane 7). This finding demonstrates that when cotranslated in vitro, ROMK2-HA and rSUR2B are physically associated. Finally, to determine the relationship between the direct interaction of ROMK with rSUR2B and glibenclamide sensitivity, we assessed the ability of ROMK1-HA to coimmunoprecipitate rSUR2B (Fig. 6, lanes 8 and 9). As expected, the ROMK1-HA protein was slightly larger (~45 kDa; Fig. 6, lanes 8 and 9) than ROMK2-HA (~43 kDa; Fig. 6, lane 2). When ROMK1-HA and rSUR2B were cotranslated, the anti-HA antibody precipitated only ROMK1-HA, but not the rSUR2B protein (Fig. 6, lane 9). Thus association of ROMK and rSUR2B proteins was observed only with ROMK2-HA, the channel isoform that exhibits sensitivity to glibenclamide (Fig. 4).

**DISCUSSION**

We report here the cloning of a rat homolog of SUR2B, rSUR2B. The predicted amino acid sequence for rSUR2B is 98.7% and 96.6% identical to mouse and human SUR2B, respectively. The proposed Walker A and B consensus sites and several additional motifs (PKA and PKC phosphorylation sites, glycosylation sites) identified in other SUR isoforms are conserved in rSUR2B. The wide expression of rSUR2B transcripts in all of the tested tissues is also in agreement with the expression pattern determined for mouse SUR2B, confirming the cloning of rat member of the SUR family. The present study provides strong evidence that the renal K<sub>ATP</sub> channel responsible for K<sup>+</sup> secretion in TAL cells and the principal cells of the CCD is comprised of ROMK2 and rSUR2B. First, the expression of ROMK2 (7, 38) and rSUR2B (Fig. 3; Ref. 6) transcripts and protein overlap in the CTAL and CCD. Second, coexpression of ROMK2 and rSUR2B in X. laevis oocytes generates whole-cell K<sup>+</sup> currents that are inhibited by glibenclamide (Figs. 4 and 5) with an EC<sub>50</sub> of 185 µM (Fig. 5). A comparison of the glibenclamide sensitivities...
of ROMK coexpressed with SUR2B (from Fig. 5) or CFTR (from Refs. 25, 31) and the native K\textsubscript{ATP} channels in rat TAL (36, 37) and principal (36) cells is shown in Fig. 7. The striking concordance in the sensitivities to glibenclamide of whole cell K\textsuperscript{+} currents in ROMK2 + rSUR2B cojected oocytes and that of native renal K\textsubscript{ATP} channels measured by inside-out patches [EC\textsubscript{50} = 150 µM (36); Fig. 7, native TAL] strongly supports the hypothesis that ROMK2 and SUR2B encode the renal secretory K\textsubscript{ATP} channel. Third, the in vitro binding assays (Fig. 6) show direct physical association of ROMK and rSUR2B and that this association of subunits is required for glibenclamide sensitivity. This is consistent with the direct association of Kir + SUR in forming K\textsubscript{ATP} channels in other tissues (22).

The ROMK2-rSUR2B complex can be communoprecipitated in the presence (Fig. 6) and absence (data not shown) of microsomal membranes, suggesting that the glycosylated forms of ROMK2 or rSUR2B are not required for the heteromultimeric complex formation. The results of the communoprecipitation studies in the in vitro translation assay also imply that an intermediate protein(s) is not necessary for the ROMK2 + rSUR2B channel subunit assembly. In contrast to ROMK2, whole cell K\textsuperscript{+} currents in ROMK1 + rSUR2B or ROMK3 + rSUR2B cojected oocytes exhibited no significant sensitivity to glibenclamide (Fig. 5), indicating that among the three known ROMK isoforms in rat kidney only ROMK2 can coassemble with rSUR2B.

Interestingly, ROMK2 is the most widely expressed of the channel isoforms along the distal nephron and collecting duct, being absent only in the outer medullary collecting duct (7). Because ROMK1 and ROMK3 differ from ROMK2 only in the NH\textsubscript{2}-terminal regions of the channel, it is likely that the NH\textsubscript{2}-terminal extensions of ROMK1 and ROMK3. In support of this hypothesis, Reimann et al. (30) showed that NH\textsubscript{2}-terminal deletions of Kir6.2 abolish high-affinity sulfonylurea block, implying that the NH\textsubscript{2}-terminal regions of Kir6.2 are involved in coupling to the SUR subunit. Several other studies have also demonstrated that the NH\textsubscript{2}-terminal domain of Kir6.2 determines the gating properties and biochemical interaction between the two subunits (5, 8, 11, 12, 19) of this K\textsubscript{ATP} channel. We are currently examining this issue for ROMK2 + rSUR2B interactions.

Several recent studies (2, 15–18) and reviews (1, 43) have suggested that the different SUR isoforms can explain the varying glibenclamide sensitivity found in K\textsubscript{ATP} channel from a variety of tissues. For example, Kir6.2 coexpressed with either SUR1 or SUR2A in mammalian cells exhibit EC\textsubscript{50} values for glibenclamide of 9 and 350 nM, respectively. The present results indicate, however, that the associated Kir subunit can also affect glibenclamide sensitivity. When SUR2B is coexpressed with Kir6.1, 3 µM glibenclamide completely abolishes channel activity (40), whereas this concentration would have negligible effect on ROMK2 + rSUR2B (Fig. 5). Thus both the SUR isoforms and the associated Kir subunits of K\textsubscript{ATP} channels determine the glibenclamide sensitivity, a finding also consistent with direct interaction of SUR with the Kir subunit.

Although CFTR is also expressed at the apical regions of distal nephron segments (9) and forms glibenclamide-sensitive K\textsuperscript{+} channels when expressed with either ROMK1 (31) or ROMK2 (24, 25) in oocytes, two observations suggest that CFTR may not form the renal epithelial K\textsubscript{ATP} channel in CTAL and CCD. First, the glibenclamide sensitivity of K\textsuperscript{+} channels in ROMK + CFTR cojected X. laevis oocytes [ROMK1 + CFTR, EC\textsubscript{50} = 33 µM (31); ROMK2 + CFTR, EC\textsubscript{50} = 2.0 µM (24); Fig. 7] is significantly higher than for the native K\textsubscript{ATP} channels in renal cells [EC\textsubscript{50} = 150 µM (36)]. Second, CFTR transcripts appear to be predominantly expressed in intercalated cells in the CCD (33), whereas ROMK is expressed only in the principal cells (38). This does not exclude the real possibility that CFTR regulates the function of ROMK in CTAL in some other fashion as it does with other channels (13, 14). Interestingly, we did not find rSUR2B in the rat MTAL (Fig. 3), whereas CFTR (or its truncated isoform; TNR-CFTR (27)) is expressed in this nephron segment. Because neither SUR1 (6) nor SUR2A (Fig. 2; Ref. 6) is expressed in the kidney, the low-conductance K\textsubscript{ATP} channel in the MTAL is either formed by an unidentified SUR or by CFTR. The latter would suggest a relatively higher (low µM) glibenclamide sensitivity of the MTAL K\textsubscript{ATP} compared with that in the CTAL (36).

Finally, K\textsubscript{ATP} channels, in parallel with Na\textsuperscript{+}-K\textsuperscript{+} ATPase, have been suggested to play an important role in K\textsuperscript{+} recycling across basolateral membranes of rabbit (34, 26) and amphibian proximal tubule (28). The expression of SUR2B in rat proximal tubules raises the possibility that this SUR in association with an unidentified Kir subunit forms the basolateral K\textsubscript{ATP} channel in this nephron segment.

In conclusion, the present study provides strong evidence that the small-conductance, secretory K\textsubscript{ATP} channel in renal CTAL and principal cells of the CCD may be comprised of ROMK2 and SUR2B, consistent with the proposed heteromultimeric formation of K\textsubscript{ATP} channels in nonrenal cells.

The nucleotide sequence reported in this manuscript has been submitted to the GenBank with accession number AF019628.

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