An Amino Acid Triplet in the NH₂ Terminus of Rat ROMK1 Determines Interaction with SUR2B*

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ATP-regulated (K\textsubscript{ATP}) channels are formed by an inward rectifier pore-forming subunit (Kir) and a sulfonylurea (glibenclamide)-binding protein, a member of the ATP binding cassette family (sulfonylurea receptor (SUR) or cystic fibrosis transmembrane conductance regulator). The latter is required to confer glibenclamide sensitivity to K\textsubscript{ATP} channels. In the mammalian kidney ROMK1–3 are components of K\textsubscript{ATP} channels that mediate K\textsuperscript{+} secretion into urine. ROMK1 and ROMK3 splice variants share the core polypeptide of ROMK2 but also have distinct NH₂-terminal extensions of 19 and 26 amino acids, respectively. The SUR2B is also expressed in rat kidney tubules and may combine with Kir1.1 to form renal K\textsubscript{ATP} channels. Our previous studies showed that co-expression of ROMK2, but not ROMK1 or ROMK3, with rat SUR2B in oocytes generated glibenclamide-sensitive K\textsuperscript{+} currents. These data suggest that the NH₂-terminal extensions in both ROMK1 and ROMK3 block ROMK-SUR2B interaction. Seven amino acids in the NH₂-terminal extensions of ROMK1 and ROMK3 are identical (amino acids 13–19 in ROMK1 and 20–26 in ROMK3) and may determine ROMK-SUR2B interaction. We constructed a series of hemagglutinin-tagged ROMK1 NH₂-terminal deletion and substitution mutants and examined glibenclamide-sensitive K\textsuperscript{+} currents in oocytes when co-expressed with SUR2B. These studies identified an amino acid triplet “IRA” within the conserved segment in the NH₂ terminus of ROMK1 and ROMK3 that blocks the ability of SUR2B to confer glibenclamide sensitivity to the expressed K\textsuperscript{+} currents. The position of this triplet in the ROMK1 NH₂-terminal extension is also important for the ROMK-SUR2B interactions. In vitro co-translation and immunoprecipitation studies with hemagglutinin-tagged ROMK mutants and SUR2B indicated that direct interaction between these two proteins is required for glibenclamide sensitivity of induced K\textsuperscript{+} currents in oocytes. These results suggest that the IRA triplet in the NH₂-terminal extensions of both ROMK1 and ROMK3 plays a key role in subunit assembly of the renal secretary K\textsubscript{ATP} channel.

ATP-regulated inwardly rectifying K\textsuperscript{+} (K\textsubscript{ATP}) channels are widely expressed in excitable cells (1–3) and kidney (4, 5) where they couple cell metabolism to electrical activity or K\textsuperscript{+} secretion. These channels are formed by octameric complexes of two types of subunits: a pore-forming subunit, the inwardly rectifying K\textsuperscript{+} channel (Kir6.1, Kir6.2, or Kir1.1), and a regulatory subunit, the sulfonylurea receptor (SUR) or the cystic fibrosis transmembrane conductance regulator (CFTR), members of the ATP binding cassette transporter protein family (3, 6–9). Sensitivity of K\textsubscript{ATP} Channel K\textsuperscript{+} currents to sulfonylurea drugs (e.g. glibenclamide) requires the associated SUR or CFTR subunit (9–11). Three isoforms of SUR have been cloned and are important for the regulation of physiological and pharmacological functions of K\textsubscript{ATP} channels (2, 10, 12, 13). SUR1 associates with Kir6.2 to form the neuronal/pancreatic β-cell-type K\textsubscript{ATP} channel; SUR2A and SUR2B, splice variants of a single gene, form either the cardiac-type (SUR2A/Kir6.2) or the vascular smooth muscle-type (SUR2B/Kir6.1 or Kir6.2) K\textsubscript{ATP} channels.

Renal K\textsubscript{ATP} channels have been identified in the apical membranes of the thick ascending limb of the loop of Henle (TAL) and principal cells of the cortical collecting duct where they mediate K\textsuperscript{+} secretion (4, 5). These K\textsubscript{ATP} channels appear to be formed by association of ROMK (Kir1.1) (5, 14) and CFTR (9, 11) and/or SUR2B (15). Three rat ROMK splice variants (ROMK1–3) are expressed in rat kidney and are identical except for distinct NH₂-terminal extensions of 19 and 26 amino acids in ROMK1 and ROMK3, respectively (16–18). We recently cloned SUR2B from rat kidney and showed that it was expressed in TAL and cortical collecting duct, similar to the localization of ROMK2 (15). Co-expression of ROMK2 with SUR2B in Xenopus laevis oocytes generated glibenclamide-sensitive K\textsuperscript{+} currents, indicating that SUR2B could be involved in forming and regulating renal K\textsubscript{ATP} channels (15). Surprisingly neither ROMK1 nor ROMK3 formed glibenclamide-sensitive K\textsuperscript{+} currents when co-expressed with SUR2B (15), suggesting that the unique NH₂-terminal extensions of ROMK1 and ROMK3 inhibited interactions with SUR2B. In the present study we evaluated the role of the unique NH₂-terminal amino acids of ROMK1 in determining interactions with SUR2B using electrophysiology and co-immunoprecipitation methods.

MATERIALS AND METHODS

Construction of HA-tagged Mutant ROMK1 and SUR2B—The hemagglutinin (HA) tag was introduced into the 3’-end of the ROMK cDNA just before the stop codon by polymerase chain reaction using ROMK1/pSPORT1 and ROMK2/pSPORT1 as templates with primers

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1 The abbreviations used are: K\textsubscript{ATP}, ATP-regulated inwardly rectifying K\textsuperscript{+}; Kir, sulfonylurea receptor; CFTR, cystic fibrosis transmembrane conductance regulator; TAL, thick ascending limb of the loop of Henle; HA, hemagglutinin; ROMK, rat outer medullary K\textsuperscript{+} channel.
Comparison of ROMK1, ROMK2, and ROMK3 NH2 termini. The NH2 terminus of ROMK2 is represented by a black bar and is shared by all ROMK splice variants. The ROMK1 and ROMK3 NH2 termini are 19 and 26 amino acids longer, respectively, than the ROMK2 NH2 terminus. The NH2-terminal extensions of ROMK1 and ROMK3 are shown by single-letter amino acid codes. The dashed lines indicate the 7-amino acid segment shared by ROMK1 and ROMK3. The first 12 amino acids in ROMK1 and 19 in ROMK3 are unique.

The NH2-terminal extension of ROMK1-HA were removed. Constructs R1-N14V-HA, and R1-N15V-HA are ROMK1-HA mutants where amino acids 14–15 replaced with valine residues), and R1-N(16Me2SO was added to the control bath solution.

In Vitro Translation of ROMK1, ROMK1 Mutants, or ROMK2 with SUR2B—In vitro translation at 22°C with external K+ concentration of 1 mM. As indicated previously (15). The in vitro-translated reaction mixtures were washed twice with ice-cold phosphate-buffered saline buffer and lyzed in a buffer containing 20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (v/v) at 4°C for 20 min followed by centrifugation at 10,000 × g for 15 min to obtain supernatants. Proteins were immunoprecipitated by incubation of supernatants with anti-HA monoclonal antibody (clone 12CA5, Roche Molecular Biochemicals) and protein A-Sepharose at 4°C for 20 min followed by overnight incubation with protein A-Sepharose at 4°C. The proteins were then recovered by incubation of protein A with 2× Laemmli SDS sample buffer at 55°C for 15 min and separated by an 8% SDS-polyacrylamide gel. The [35S]methionine-labeled proteins were visualized by autoradiography.

RESULTS

Heterologous Expression of ROMK1, ROMK2, and ROMK1 Truncation Mutants with SUR2B in X. laevis Oocytes—To determine whether SUR2B forms glibenclamide-sensitive K+ currents with ROMK1 truncation mutants, we first evaluated Ba2+-sensitive K+ currents in oocytes co-injected with cRNA transcribed from SUR2B and ROMK1-HA, ROMK2-HA, or the HA-tagged ROMK1 mutant:3

Fractional K+ currents (15). This observation was confirmed in the present study. Glibenclamide reduced whole cell K+ currents in oocytes co-injected with ROMK2 and SUR2B (n = 41): fractional K+ currents with glibenclamide compared with controls (I/I0) were 0.47 ± 0.06, p < 0.01 (Figs. 3, B and C). Co-injection of ROMK1 wild type or the ROMK1 truncation mutants R1-NΔ5-HA, R1-NΔ10-HA, or R1-NΔ12-HA with SUR2B did not give rise to glibenclamide-sensitive K+ currents (Fig. 3, A and C). Fractional K+ currents (I/I0) after addition of 0.2 mM glibenclamide with ROMK1 (n = 8), R1-NΔ5-HA (n = 4), R1-NΔ10-HA (n = 4), and R1-NΔ12-HA (n = 7) co-expressed
FIG. 3. Glibenclamide sensitivity of ROMK1, ROMK1 truncation mutants, and ROMK2 co-expressed with SUR2B. ROMK1 (or ROMK2) wild type or ROMK1 mutant construct cRNAs (5 ng) were co-injected with SUR2B cRNA (50 ng). Whole cell currents were monitored in X. laevis oocytes by two-electrode voltage clamp with 1 mM bath K\textsuperscript{+}, and all currents could be abolished by 5 mM Ba\textsuperscript{2+}. The oocytes were voltage-clamped at \(-100\) mV and pulsed in steps of 20 mV for 50 ms over the range of \(-160\) to \(+40\) mV. A and B show current tracings in the absence and presence of 0.2 mM glibenclamide. C, summary of fractional K\textsuperscript{+} currents with glibenclamide compared with control without glibenclamide (I/I_{0}, mean \pm S.E.). * indicates p < 0.05 compared with control K\textsuperscript{+} currents (I/I_{0}) in oocytes co-injected with ROMK1 and SUR2B. ** indicates p < 0.01 compared with control K\textsuperscript{+} currents (I/I_{0}) in oocytes co-injected with ROMK1 and SUR2B. (Only R1-N\textsubscript{15}-SUR2B currents with glibenclamide sensitivity were analyzed.) Above each bar graph is a representation of the specific ROMK1 construct. R2, represents the core, 372-amino acid, region identical in all ROMK splice variants. aa, amino acids.
with SUR2B were 0.98 ± 0.10, 1.13 ± 0.27, 1.07 ± 0.36, and 0.86 ± 0.23, respectively. In contrast, co-expression of R1-NΔ15 with SUR2B generated glibenclamide-sensitive K⁺ currents in 57% of injected oocytes (n = 7); I/I₀ was 0.54 ± 0.09, p < 0.05 (Fig. 3, B and C). Co-injection of the further truncated mutants R1-NΔ16 or R1-NΔ17 with SUR2B generated glibenclamide-sensitive K⁺ currents in all oocytes; I/I₀ values were 0.48 ± 0.13 (n = 8) and 0.51 ± 0.13 (n = 4), p < 0.01 (Fig. 3, B and C).

In Vitro Co-translation and Co-immunoprecipitation of ROMK1 Truncation Mutants and SUR2B—We previously showed that the functional and biochemical interactions between ROMK2 or ROMK1 and SUR2B are directly correlated (15). To assess whether the functional interaction of the HA-tagged ROMK1 truncation mutants with SUR2B also indicates direct interaction between the channel subunits, the proteins were translated in vitro and immunoprecipitated with anti-HA antibody. In vitro translation of ROMK1-HA, ROMK2-HA, and the HA-tagged ROMK1 truncation mutants in the presence of canine pancreatic microsomal membranes showed the expected molecular mass bands between 42 and 45 kDa. Fig. 4 shows that co-translation of ROMK1-HA, ROMK2-HA, or the HA-tagged ROMK1 truncation mutants with SUR2B produced two distinct bands in each lane: the 174-kDa band representing SUR2B and a band between 43 and 45 kDa representing the HA-tagged ROMK protein. To determine whether SUR2B co-precipitated with the ROMK proteins, we used anti-HA antibody to immunoprecipitate ROMK1-HA, ROMK2-HA, or the HA-tagged ROMK1 truncation mutants and then resolved proteins by SDS-polyacrylamide gel electrophoresis. The SUR2B protein did not co-immunoprecipitate with ROMK1-HA or the ROMK1-HA truncation mutants R1-NΔ5-HA, R1-NΔ10-HA, and R1-NΔ12-HA (Fig. 5). In contrast, immunoprecipitation of R1-NΔ16-HA, R1-NΔ17-HA, or ROMK2-HA brought down the SUR2B protein (Fig. 5). When R1-NΔ15-HA was co-translated with SUR2B and the proteins were immunoprecipitated with anti-HA antibody, a mixed result was obtained: sometimes the anti-HA precipitated a complex of R1-NΔ15-HA and SUR2B proteins, but other times it just precipitated the R1-NΔ15-HA protein (data not shown). This is consistent with the variable glibenclamide sensitivity seen with this mutant construct. Thus, our biochemical data are consistent with the electrophysiological results: glibenclamide sensitivity occurs only when ROMK and SUR2B directly associate, and amino acids in the extended NH₂ terminus of ROMK1 inhibit its association with SUR2B. Specifically, these results indicate that amino acids 13–15 in the NH₂ terminus of ROMK1 inhibit the interaction between ROMK1 and SUR2B.

Functional Expression of Amino Acids 13–19 ROMK1 Mutants and SUR2B in Oocytes—To assess the role of amino acids 13–19 in the ROMK1 NH₂ terminus in determining the interaction between ROMK1 and SUR2B, we produced seven additional mutants (Fig. 2B): R1-NΔ(13–19)-HA (deletion of amino acids 13–19), R1-NS-HA (switching amino acids 13–19 to the start of the NH₂ terminus), R1-N(13–15)V-VHA (replacing amino acids 13–15 with valine), R1-N(16–18)V-HA (replacing amino acids 16–18 with valine), R1-N13V-HA (replacing amino acid 13 with valine), R1-N14V-HA (replacing amino acid 14 with valine), and R1-N15V-HA (replacing amino acid 15 with valine). Oocytes co-injected with R1-NΔ(13–19)-HA and SUR2B exhibited glibenclamide-sensitive K⁺ currents, confirming that amino acids 13–19 block the interaction between SUR2B and ROMK1. The fractional K⁺ current (I/I₀) after 0.2 mM glibenclamide (n = 5) was 0.59 ± 0.18, p < 0.01 (Fig. 6, A and B). This result also confirmed that the first 12 amino acids of the ROMK1 NH₂ terminus do not affect the ROMK1 and SUR2B interaction. Interestingly we also observed glibenclamide-sensitive K⁺ currents in oocytes injected with R1-NS-HA and SUR2B: I/I₀ was 0.55 ± 0.07, p < 0.01 (n = 9) (Fig. 6, A and C). Thus, the position of amino acids 13–19 is also crucial for ROMK and SUR2B interaction. To determine which of these 7

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**Fig. 4.** In vitro co-translation of ROMK1, ROMK1 truncation mutants, and ROMK2 with SUR2B. ROMK construct cDNA was co-translated in vitro with SUR2B cDNA in the presence of [³⁵S]methionine and canine microsomal membranes. Proteins were analyzed by 8% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Core molecular masses of SUR2B, ROMK1-HA, HA-tagged ROMK1 truncation mutants, and ROMK2-HA were 174, 45, 45–43, and 43 kDa, respectively. Above each lane is a representation of the specific ROMK1 construct. R2, see Fig. 2; aa, amino acids.

**Fig. 5.** Co-immunoprecipitation of SUR2B with HA-tagged ROMK constructs. HA-tagged ROMK constructs and SUR2B were co-translated in vitro in the presence of [³⁵S]methionine and canine microsomal membranes. Resultant proteins were immunoprecipitated (IP) using anti-HA antibody, and immunoprecipitated proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The upper 174-kDa bands represent SUR2B protein, and the ~45-kDa lower bands are HA-tagged ROMK. Above each lane is a representation of the specific ROMK1 construct. R2, see Fig. 2; aa, amino acids.
FIG. 6. Role of the NH₂-terminal 7-
amino acid segment (see Fig. 1) in
ROMK1 and SUR2B functional inter-
actions. Experimental details are the
same as in Fig. 3. A and B show current
tracings in the absence and presence of
0.2 mM glibenclamide. C, summary of
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compared with control without gliben-
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and SUR2B. Above each bar graph is a
representation of the specific ROMK1
construct. R2, see Fig. 2; aa, amino acids.
amino acids (IRALTER) inhibits the interaction between ROMK1 and SUR2B and thus glibenclamide sensitivity, we assessed glibenclamide-sensitive K⁺ currents in oocytes co-injected with SUR2B and either R1-N(13–15)V-HA or R1-N(16–18)V-HA (Fig. 6, A and C). We found that R1-N(13–15)V-HA, but not R1-N(16–18)V-HA, exhibited sulfonylurea sensitivity: I/I₀ was 0.41 ± 0.23 (n = 8; p < 0.05) and 0.91 ± 0.26 (n = 7; p > 0.05), respectively. Thus amino acids 13–15 (IRA) in the ROMK1 NH₂-terminal extension are crucial for inhibiting the interaction between ROMK1 and SUR2B. We also generated single valine mutants for each of the amino acids, 13–15 (IRA), and examined the glibenclamide sensitivity. Co-expression of each single mutant with SUR2B produced glibenclamide-sensitive K⁺ currents. I/I₀ values from R1-N13V-HA, R1-N14V-HA, or R1-N15V-HA with SUR2B were 0.51 ± 0.11 (n = 8), 0.39 ± 0.10 (n = 8), and 0.50 ± 0.09 (n = 10; p < 0.01), respectively (Fig. 6, B and C).

In Vitro Co-Translation and Co-immunoprecipitation of Individual ROMK1 Mutants with SUR2B—Fig. 7 shows the results of the in vitro co-translation of R1-NΔ(13–19)-HA or R1-NS-HA and SUR2B performed in the presence of canine pancreatic microsomal membranes. Anti-HA antibody co-immunoprecipitated each of these ROMK1 mutants together with SUR2B. When R1-N(13–15)V-HA or R1-N(16–18)V-HA and SUR2B were co-translated, SUR2B was co-immunoprecipitated with R1-N(13–15)V-HA but not with R1-N(16–18)V-HA (Fig. 8). These results are consistent with the glibenclamide sensitivity of the K⁺ currents observed when these mutants were co-expressed with SUR2B. Moreover, they also confirm that the amino acid triplet (amino acids 13–15, IRA) in ROMK1 plays a key role determining the assembly of ROMK1 with SUR2B, and thereby conferring glibenclamide sensitivity to the Kₐ₅₆ channel.

**DISCUSSION**

The present study defines the molecular site on ROMK1 that inhibits its interaction with SUR2B to form a sulfonylurea-sensitive K⁺ channel. Our previous study demonstrated that ROMK2, but not ROMK1 or ROMK3, formed a glibenclamide-sensitive K⁺ channel when co-expressed with SUR2B (15). ROMK1 and ROMK3 are alternatively spliced forms of the renal ATP-regulated K⁺ channel that contain NH₂-terminal extensions of 19 and 26 amino acid residues, respectively. Compared with ROMK2, ROMK1 and ROMK3 share a 7-amino acid segment just preceding the ROMK2 initiator methionine (Fig. 1) and we had proposed that this segment could be involved in blocking the interaction of ROMK and SUR2B (15). In the present study we confirmed that ROMK2, but not ROMK1, formed a glibenclamide-sensitive K⁺ channel when co-expressed with SUR2B, and we have now identified three amino acids (in ROMK1 amino acids 15–17, IRA) that inhibit the interaction with SUR2B and thus render the ROMK1 channel insensitive to glibenclamide. This amino acid triplet is contained within the 7-amino acid region, 13–19, which is identical to amino acids 20–26 in ROMK3 and could also account for the lack of ROMK3 interaction with SUR2B.

Since glibenclamide sensitivity was only observed when ROMK co-immunoprecipitated with SUR2B, sulfonylurea sensitivity requires direct interaction between these two proteins. While we do not know the stoichiometry of ROMK-SUR2B to form glibenclamide-sensitive K⁺ channels in oocytes, previous studies of SUR1 and the ATP-sensitive K⁺ channel Kir6.2 demonstrated a one-to-one stoichiometry in a 4:4 hetero-octamer (3). The NH₂ terminus of Kir6.2 has been suggested to provide a site for coupling to the sulfonylurea receptor (21). Future studies will be required to investigate what regions and amino acid residues in ROMK2 play a similar role in the interaction with SUR2B. If a similar region in ROMK2 is required for interacting with SUR2B, then it is possible that the inhibitory “IRA” triplet in ROMK1 interferes with this NH₂-terminal SUR2B binding site by either binding to this site...
region or altering its structure. Interestingly the relative position within the NH2 terminus of this amino acid triplet appears to be critical since transposing it to the beginning of the NH2 terminus allows ROMK1 interaction with SUR2B.

It is generally accepted that ROMK forms the small conductance ATP-regulated and glibenclamide-sensitive K+ channel in distal nephron segments of the mammalian kidney mediating K+ secretion (5, 14). When ROMK is expressed alone in X. laevis oocytes it is not sensitive to glibenclamide (9, 11, 15), indicating that it must be co-associated with a sulfonylurea receptor protein in native tissue. In this regard, ROMK2 has been shown to form glibenclamide-sensitive K+ currents when co-expressed with the CFTR (9, 11) and with the sulfonylurea receptor proteins SUR1 (22) and SUR2B (Ref. 15 and Fig. 3, B and C). CFTR, like the sulfonylurea receptor, is also a member of the ATP binding cassette transporter gene family and has been shown to impart glibenclamide sensitivity to other channels (23). SUR1 is not expressed in kidney epithelia, but both SUR2B (15, 24) and CFTR transcripts and protein (25) are found in several distal nephron segments that also express ROMK isoforms (17). Thus, both CFTR and SUR2B may contribute to forming ATP-regulated, small conductance K+ secretory channels in the kidney.

While ROMK2-SUR2B and ROMK2-CFTR can form glibenclamide-sensitive K+ currents, CFTR, but not SUR2B, can also impart sulfonylurea sensitivity to K+ currents when co-expressed with ROMK1. ROMK2 is expressed in all distal nephron segments from the TAL to the cortical collecting duct, expressed with ROMK1. ROMK2 is expressed in all distal nephron segments from the TAL to the cortical collecting duct, while ROMK3 is found only in the collecting duct and ROMK3 nephron segments from the TAL to the cortical collecting duct, expressed with ROMK1. ROMK2 is expressed in all distal nephron segments from the TAL to the cortical collecting duct, while ROMK3 is found only in the collecting duct and ROMK3 nephron segments from the TAL to the cortical collecting duct, expressed with ROMK1.

In conclusion, our results demonstrate that the specific location of amino acid triplet IRA (residues 13–15) in the NH2-terminal extension of ROMK1 (and likely ROMK3) plays a key role in the inhibition of subunit assembly of the renal secretary K\textsubscript{ATP} channel ROMK with SUR2B and can account for the lack of glibenclamide sensitivity when SUR2B is co-expressed with ROMK1 or ROMK3.

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