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Cloning and functional expression of a novel isoform of ROMK inwardly rectifying ATP-dependent K^+ channel, ROMK6 (Kir1.1f)

Chikako Kondo[a,b], Shojiro Isomoto[a], Shigeto Matsumoto[a,b], Mitsuhiko Yamada[a], Yoshiyuki Horio[a], Shizuya Yamashita[b], Kaoru Takemura-Kameda[b], Yuji Matsuzawa[b], Yoshihisa Kurachi[a,*]

[a]*Department of Pharmacology II, Faculty of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565, Japan*
[b]*Internal Medicine II, Faculty of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565, Japan*

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Abstract We have identified from rat kidney a novel isoform of ROMK/Kir1.1, designated ROMK6/Kir1.1f. ROMK6 was nearly identical to ROMK1, but possessed an 122-bp insertion in the 5′ region. Its deduced amino acid sequence was shorter by 19 amino acids than that of ROMK1 in the amino-terminus. Unlike other previously reported ROMK isoforms, ROMK6 mRNA was ubiquitously expressed in various tissues, including kidney, brain, heart, liver, pancreas and skeletal muscle. *Xenopus* oocytes injected with ROMK6 cRNA expressed a Ba^{2+}-sensitive weakly inwardly rectifying K^+ current. These results indicate that ROMK6 is a novel functional K^+ channel and might be involved in K^+ secretion in various tissues.

Key words: Cloning; Inwardly rectifying K^+ channel; ROMK channel; Splice variant; Reverse transcription-polymerase chain reaction; *Xenopus* oocyte

1. Introduction

Inwardly rectifying K^+ channels play significant roles in controlling excitability and secretion of K^+ ions in various types of cells. The first cDNA encoding an inwardly rectifying K^+ channel has been isolated from rat renal outer medulla by expression cloning [1] and designated ROMK1 (Kir1.1a). Immunohistochemical analyses showed that ROMK1 protein was localized to the apical membrane of renal cortical collecting duct epithelial cells [2]. ROMK1 expressed in *Xenopus* oocytes forms a weakly inwardly rectifying K^+ channel current with a single channel conductance of 35–45 pS with 90 mM extracellular K^+. The channel requires intracellular Mg-ATP to open, exhibits a high open probability at various potentials, and is inhibited by intracellular H^+. These properties are comparable to those of the low-conductance K^+ channel electrophysiologically identified in the apical membrane of cortical collecting duct cells [1,3–8].

Recent studies have shown that the human ROMK gene is composed of at least five exons, while exon 3 has not been reported in the rat ROMK gene, and produces divergent mRNAs as a result of alternative splicing at their 5′ regions [9–11]. So far, five isoforms of ROMK mRNAs in human have been identified and designated ROMK1–5 (Kir1.1a–e) [9,12]. In rat, so far, three isoforms (ROMK1–3) have been isolated [13]. This divergence of ROMK mRNAs may result in the formation of channel subunit proteins with variable lengths of amino (N)-termini. It was also shown that these ROMK isoforms were differentially expressed among different segments of renal tubules [13], where they might play distinct roles in controlling K^+ secretion.

In the present study, we have tried to find a new member of the ROMK family from rat kidney, using the RT-PCR cloning technique. We have identified a novel ROMK isoform and designated it ROMK6 (Kir1.1f). ROMK6 expressed a weakly inwardly rectifying K^+ current in *Xenopus* oocytes. Unlike other previously reported ROMK isoforms, its mRNA was ubiquitously expressed in various tissues including kidney, brain, heart, liver, pancreas and skeletal muscle. Thus, ROMK6 might be involved in K^+ secretion in various tissues.

2. Materials and methods

2.1. Isolation of cDNAs encoding ROMK channel

Total RNA prepared from whole rat kidney was reverse transcribed using an oligo-(dT) primer. The resulting cDNA was amplified by PCR using a sense primer (nucleotides −87 to −67 in rat ROMK1; 5′-GCTTAAGATTCATTAAGGTGG-3′) paired with an antisense primer (nucleotides 1174–1194; 5′-TAGGTGGAAAAGCCACTGC-TA-3′). The PCR conditions were as follows: an initial denaturation at 94°C for 4 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 1 min, with a final extension step at 72°C for 8 min. The amplified fragments were subcloned into pBluescript II SK-plasmid (Stratagene, La Jolla, CA), and sequenced on both strands using a DNA sequencer (DSQ-1000, Shimadzu, Kyoto, Japan).

2.2. Functional expression in Xenopus oocytes and electrophysiological measurements

Each plasmid was linearized by digestion with *Kpn*I and capped cRNAs prepared with T3 RNA polymerase using a mCAP RNA capping kit (Stratagene). Manually defolliculated oocytes were injected with cRNAs dissolved in water (50 nl of 500 ng/μl). For control, the same volume of sterile water was injected. After injection, oocytes were incubated in ND96 solution at 18°C, and electrophysiological studies were undertaken 48–96 h later. Two-electrode voltage clamp experiments were carried out with an amplifier (Turbo Clamp TEC 01C, Tamm, Germany) using microelectrodes filled with 3 M KCl. Oocytes were bathed in a solution which contained 90 mM KCl, 3 mM $MgCl_2$, 5 mM HEPES, pH 7.4, and 300 μM niflumic acid to block endogenous chloride current. Oocytes were voltage-clamped at 0 mV and voltage steps of 1.2 s duration from −120 to +60 mV in 20-mV increments were applied to the cells every 5 s. Results were expressed as mean ± S.E.

2.3. Tissue distribution of rat ROMK isoforms

Distribution of ROMK isoforms in different rat tissues was determined by RT-PCR. Total RNAs from various organs were extracted by the guanidine thiocyanate method and were reverse transcribed using an oligo-(dT) primer. The resulting cDNAs were amplified in the PCR using a ROMK-specific sense primer (nucleotides 13–30 in rat ROMK1; 5′-GAACGGAGTGTGTTCAGA-3′) paired with

*Corresponding author. Fax: (81) (6) 879-3519.
E-mail: ykurachi@pharma2.med.osaka-u.ac.jp

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an antisense primer (nucleotides 536–553; 5′-GTCTAGAGATCTTGGCTA-3′). The RT-PCR products were then amplified by second PCR using a sense primer (nucleotides 18–35; 5′-GAGTGTGTTCAGAGTGCT-3′) and an antisense primer (nucleotides 218–237; 5′-GTACCTCCATTTCAGGTCCA-3′). Amplified fragments were electrophoretically fractionated on 2% agarose gels. RT-PCR products from kidney and skeletal muscle were subcloned into pCR vector (Invitrogen, San Diego, CA), and were sequenced as described above to confirm the sequences of an ROMK isoform.

3. Results

The RT-PCR using the primers for ROMK1 (see Section 2) of rat kidney total mRNAs yielded two transcripts of 1281 bp and 1403 bp. The transcript of 1403 bp was less visible than that of 1281 bp. The sequence analysis showed that the clone of 1281 bp was identical to rat ROMK1 except for three bp substitutions at nucleotide positions 235, 555 and 1104. All of them were C in rat ROMK1 [1], and T in the 1281-bp clone. The same substitutions at corresponding sites were also detected in the 1403-bp clone. These substitutions might be due to gene polymorphism in rat ROMK gene, but did not cause any alteration in the deduced amino acid sequence of the 1281-bp clone from that of rat ROMK1. Such substitutions have also been demonstrated between rat ROMK2 and ROMK2b [13].

The 1403-bp clone had an 122-bp insertion in the 5′-region between the nucleotides at positions 36 and 37 in rat ROMK1 (Fig. 1A). None of ROMK isoforms reported so far contained this 122-bp fragment in their nucleotide sequences. Therefore, we considered this 1403-bp clone to be a novel isoform of ROMKs and designated it rat ROMK6 (Kir1.1f). This result suggests that a novel exon which has not been identified earlier exists in ROMK gene.

The putative structure of the ROMK gene expected from previous and present studies on the ROMK isoforms [1,9–11] is depicted in Fig. 1B. The previous studies have indicated that the human ROMK gene is composed of five exons: four exons (exons 1–4) in the 5′-end and the core exon in the 3′-end. The present study suggests that a novel exon (exon 5) exists between exon 4 and the core exon in the rat ROMK gene. This exon has not been identified in the human ROMK gene. Thus, it might be specific to the rat gene. Because exon 3 in human ROMK gene has not been reported in the rat gene, ROMK genes both in human and rat may be composed of at least five exons: i.e. exons 1–4 and the core exon in human, and exon 1, exon 2, exon 4, exon 5 and the core exon in rat (Fig. 1B). The additional 122-bp segment found in rat ROMK6 mRNA, which may be derived from exon 5, can introduce an in-frame stop codon upstream of the ATG in the core exon of rat ROMK isoforms as indicated by

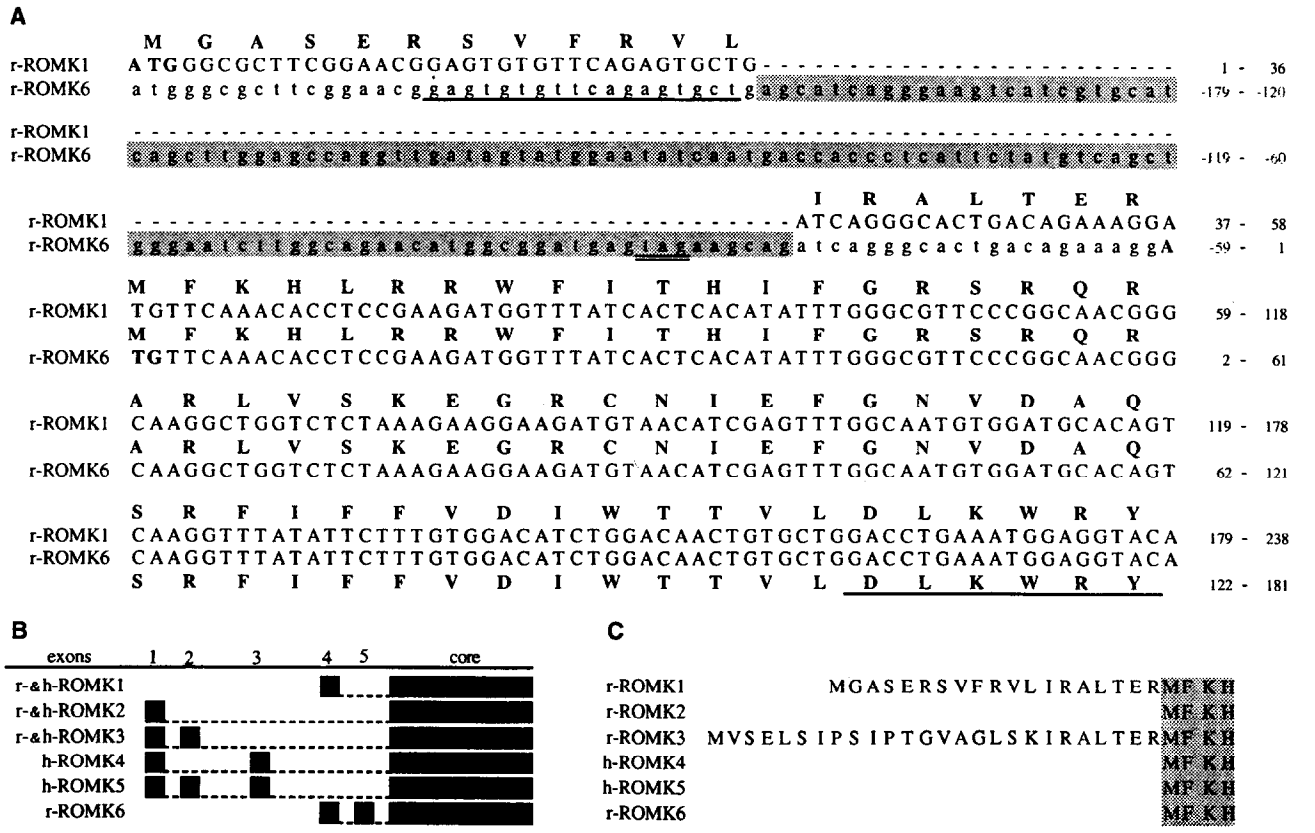

Fig. 1. Nucleotide and deduced amino acid sequences of rat ROMK cDNAs. (A) Aligned nucleotide and deduced amino acid sequences in the 5′-region of rat ROMK1 and rat ROMK6. The nucleotides of ROMK1 and ROMK6 are numbered to the right of each sequence. The termination codon is double underlined. The nucleotide sequences of sense and antisense primers for RT-PCR to determine tissue distributions of ROMK1 and ROMK6 mRNAs are underlined. ROMK6 has a nucleotide sequence identical to ROMK1 and possesses an additional 122-bp insertion, which is shaded, in the N-terminal region between nucleotide positions 36 and 37 in ROMK1. The insertion of 122 bp introduces an in-frame stop codon upstream of the ATG in the core exon of ROMK isoforms as indicated by the double bars. (B) The putative structure of rat and human ROMK genes encoding ROMK6 and previously reported ROMK isoforms. ROMK6 may possess a novel exon (exon 5) between exon 4 and a common core exon of ROMK isoforms. (C) N-terminal amino acid sequences in rat or human ROMK isoforms. The start of common amino acid sequences is shaded. ROMK6 would not contain the first 19 amino acids present in the rat ROMK1 protein.

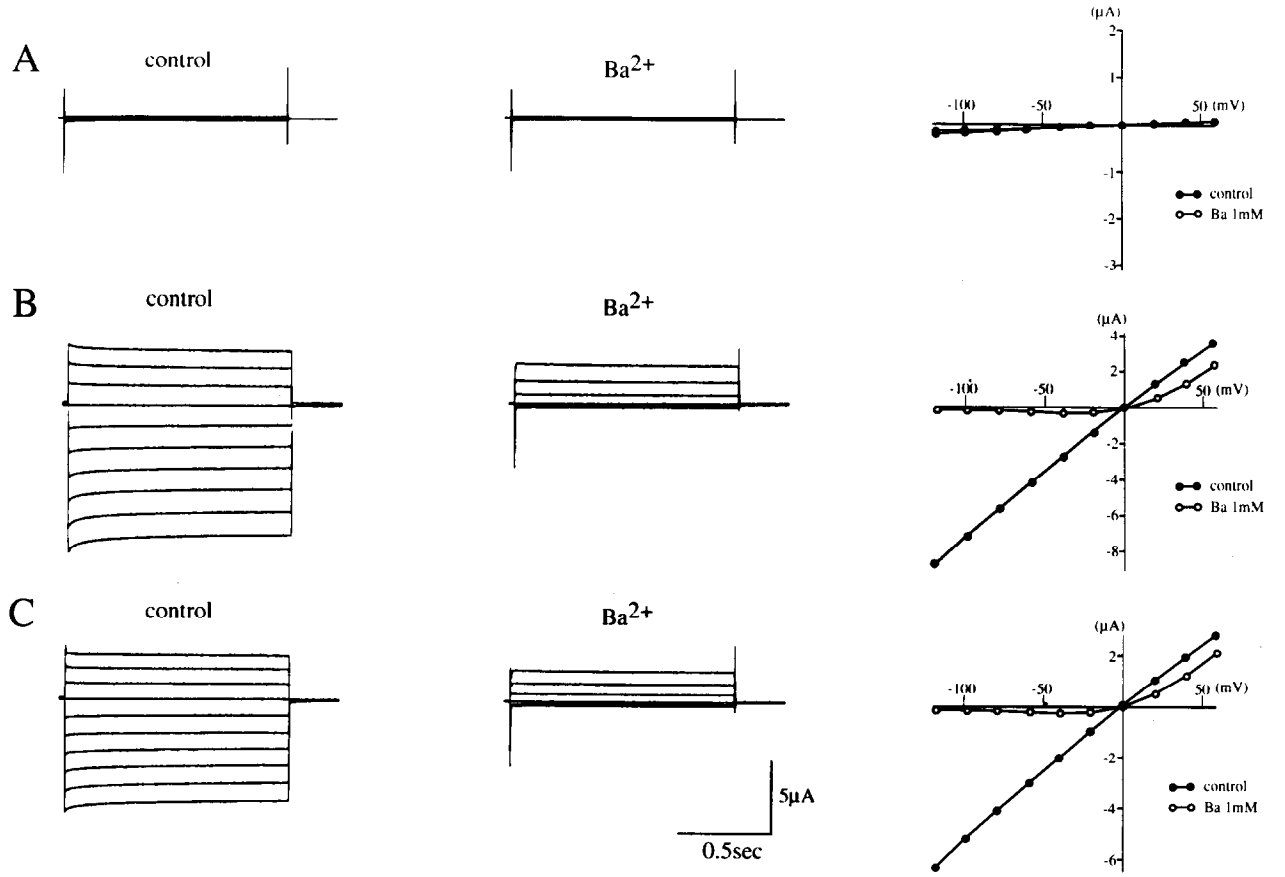

Fig. 2. Functional expression of rat ROMK1 and ROMK6 in *Xenopus* oocytes. Representative current records from *Xenopus* oocytes injected with H_2O (A), ROMK1 cRNA (B) or ROMK6 cRNA (C) in 90 mM KCl solution without (first column) and with 1 mM $BaCl_2$ (second column) are shown. The steady-state current-voltage relationships of these currents in solutions without Ba^{2+} (closed circles) and with 1 mM Ba^{2+} (open circles) are shown in the third column. Both ROMK1 and ROMK6 exhibited a weak inwardly rectifying profile, which was not observed in H_2O-injected oocytes. These inward currents were almost completely inhibited by 1 mM Ba^{2+}.

the double bars in Fig. 1A. Consequently, the deduced amino acid sequence of rat ROMK6 would be shorter by 19 amino acids than that of rat ROMK1 in the N-terminus and thus would be the same as those of rat and human ROMK2 and human ROMK4, 5 (Fig. 1C).

Representative membrane currents recorded from *Xenopus* oocytes injected with H_2O (A), rat ROMK1 cRNA (B) or ROMK6 cRNA (C) are shown in Fig. 2. ROMK6 as well as ROMK1 currents exhibited weakly inwardly rectifying K^+ currents, which were never observed in H_2O-injected oocytes. In both cases of ROMK1 and ROMK6, Ba^{2+} (1 mM) added to the bathing solution blocked the inward currents almost completely, but hardly affected the outwardly going currents at depolarized potentials. The Ba^{2+}-sensitive currents at −120 mV at the end of the voltage steps were 8.27 ± 0.23 μA ($n = 18$) in the oocytes injected with ROMK1 cRNA and 6.67 ± 0.26 μA ($n = 6$) in those with ROMK6 cRNA.

To determine tissue distributions of ROMK1 and ROMK6 mRNAs, RT-PCR assay was performed (Fig. 3). The specific primers for amplification of both ROMK1 and ROMK6 were designed to yield transcripts of 220-bp for ROMK1 and 342-bp for ROMK6. Thus, in the tissues where both ROMK1 and ROMK6 mRNAs are expressed, two transcripts of 220 bp and 342 bp can be detected. As shown in Fig. 3, ROMK1 mRNA was expressed mainly in kidney and spleen, and slightly in brain, eye, small intestine, colon and testis. On the other hand, ROMK6 mRNA distributed not only in these tissues but also in all other tissues examined: atrium, ventricle, lung, liver, pancreas and skeletal muscle. RT-PCR products with expected lengths of ROMK6 from kidney and skeletal muscle were sequenced, and the presence of the ROMK6 specific 122-bp splice was confirmed (data not shown).

4. Discussion

A major difference between ROMK6 and ROMK1 in rat was their tissue distribution. It has been reported that ROMK isoforms are predominantly expressed in kidney of both rat and human by Northern blot analysis [9–11]. The distribution of other rat ROMK isoforms (ROMK2 and ROMK3) was shown to be different from that of rat ROMK1 within kidney, but has not been examined in other tissues [13]. In human tissues, Shuck et al. [9] performed the RT-PCR assay using sense primers specific for either exon 1, exon 2, exon 4 or the core exon paired with a common antisense primer (core exon-specific), and showed that the amplicon derived from exon 1 was only expressed in kidney, that from exon 2 in kidney, brain and heart, that from exon 4 in kidney and pancreas, and that from the core exon in all tissues examined: brain, heart, kidney, liver, skeletal muscle, pancreas and spleen. However, the distribution of exon 3, the human-specific

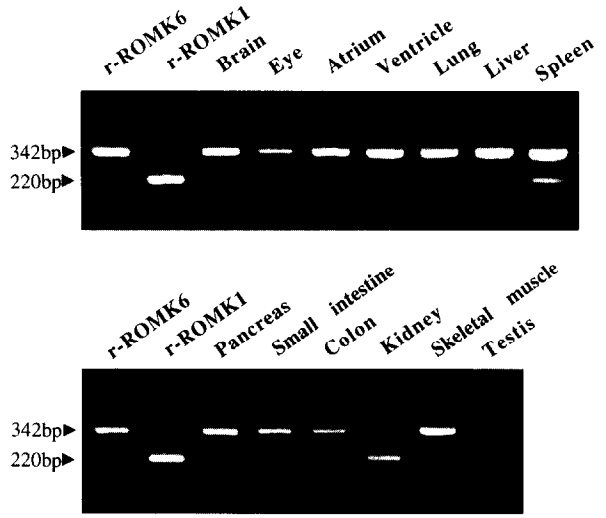

Fig. 3. RT-PCR detection of rat ROMK1 and ROMK6 mRNAs. The RT-PCR assay was performed as described in Section 2. The RT-PCR yielded visible amplified product (220 bp) of ROMK1 in mRNA of brain, eye, spleen, small intestine, colon, kidney and testis, and that (342 bp) of ROMK6 in mRNA of all tissues examined in this study as follows: brain, eye, atrium, ventricle, lung, liver, spleen, pancreas, small intestine, colon, kidney, skeletal muscle and testis.

exon, has not been examined. On the other hand, we found that the amplicon corresponding to ROMK1 (complex of exon 4 and the core exon) was detected in rat kidney, spleen and brain but not in pancreas, and that corresponding to ROMK6 (complex of exon 4, exon 5 and the core exon) was detected in all rat tissues examined. The novel exon (exon 5) found in ROMK6 has not been reported in the human ROMK gene, while exon 3 reported in the human ROMK gene has not been identified in the rat ROMK gene [9,10]. Thus, the structure of the ROMK gene may differ between rat and human. The different structure of the ROMK gene between rat and human may underlie the different tissue distribution of the various ROMK isoforms in these species.

RT-PCR for ROMK6 mRNA indicated that this clone is ubiquitously expressed in various rat tissues, whereas rat ROMK1 and human ROMK isoforms (ROMK1, ROMK2 and ROMK3) are expressed in limited tissues. Therefore, unlike other previously reported ROMK isoforms, ROMK6 might be involved in K^+ secretion not only in kidney but also in other tissues. Ubiquitous distribution of ROMK6 may also indicate the possibility that this clone expresses in such subsets of cells that distribute widely in various tissues, e.g. vascular smooth muscle, endothelial, epithelial and peripheral nerve cells.

The insertion of exon 5 introduced an in-frame stop codon upstream of the ATG within a Kozak sequence [14] in the core exon of ROMK isoforms. Consequently, the deduced protein of ROMK6 is identical to that of rat ROMK2, but is 19 and 26 amino acids shorter than those of rat ROMK1 and rat ROMK3, respectively. Human ROMK2, ROMK4 and ROMK5 also basically produce the same protein as rat ROMK2 and ROMK6 (Fig. 1B,C). Previous studies indicated that these variations in the N-terminal ends affect neither ion permeation nor channel gating in both human and rat [9,11,13]. Consistently, the K^+ currents in *Xenopus* oocytes induced by ROMK6 cRNA showed similar properties to those by ROMK1. Distributions but not electrophysiological properties are different among ROMK isoforms, suggesting that physiological roles of ROMK isoforms may be mainly dependent on their tissue distribution. Because ROMK6 is the only one which distributes ubiquitously, this clone might be unique to be involved in K^+ secretion in a wide variety of tissues, while other ROMK isoforms may play significant roles mainly in kidney.

It has been shown that expression of rat ROMK1 with a sulfonylurea receptor (SUR), a member of the ATP-binding cassette (ABC) superfamily, in *Xenopus* oocytes conferred sensitivity to sulfonylureas, such as tolbutamide, on rat ROMK1 currents [15]. In addition, McNicholas et al. [16] reported that coexpression of rat ROMK2 and the cystic fibrosis transmembrane regulator (CFTR), another member of the ABC superfamily, significantly enhanced the sensitivity of rat ROMK2 to the sulfonylureas, and concluded that ROMK2 and CFTR may reconstitute the main characteristic feature of epithelial type ATP-sensitive K^+ channels. If the CFTR/ROMK2 complex is the epithelial ATP-sensitive K^+ channels, ROMK2 should be expressed in all tissues containing epithelial cells. ROMK2, however, is expressed in a limited number of tissues at least in human, while CFTR is expressed in various epithelial cells including those of kidney, lung, pancreas and gastrointestinal tract of both human and rat [17–19]. Therefore, ROMK2 cannot be always the partner of CFTR to form the epithelial ATP-sensitive K^+ channels. Because rat ROMK6 has the deduced protein identical to that of rat ROMK2 and was expressed ubiquitously among various tissues, this clone might be a better candidate associated with CFTR to form the epithelial ATP-sensitive K^+ channels at least in rat. Further studies are needed to elucidate this possibility and other functional roles of ROMK6 in vivo.

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