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Mislocalization of K^+ channels causes the renal salt wasting in EAST/SeSAME syndrome

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ABSTRACT

The Kir4.1/Kir5.1 channel mediates basolateral K^+ recycling in renal distal tubules; this process is critical for Na^+ reabsorption at the tubules. Mutations in Kir4.1 are associated with EAST/SeSAME syndrome, a genetic disorder characterized by renal salt wasting. In this study, we found that MAGI-1 anchors Kir4.1 channels (Kir4.1 homomer and Kir4.1/Kir5.1 heteromer) and contributes to basolateral K^+ recycling. The Kir4.1 A167V mutation associated with EAST/SeSAME syndrome caused mistrafficking of the mutant channels and inhibited their expression on the basolateral surface of tubular cells. These findings suggest mislocalization of the Kir4.1 channels contributes to renal salt wasting.

Structured summary of protein interactions:
Kir5.1 and **Kir4.1** colocalize by fluorescence microscopy (View interaction)
MAGI-1 physically interacts with **Kir4.1** by anti bait coimmunoprecipitation (View interaction)
MAGI-1 physically interacts with **Kir4.1** by anti bait coimmunoprecipitation (1, 2)
MAGI-1 physically interacts with **Kir4.1** by pull down (View interaction)
Kir5.1 physically interacts with **Kir4.1** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

Genetic disorders have provided clues to the physiological functions of several proteins. EAST/SeSAME syndrome is an autosomal recessive genetic disorder characterized by a unique set of symptoms: epilepsy (seizures), ataxia, sensorineural deafness, mental retardation, and tubulopathy (electrolyte imbalance) [1,2]. Mutations in *KCNJ10* have been associated with this syndrome. *KCNJ10* encodes Kir4.1, a member of the K^+ channel family known as inwardly rectifying K^+ (Kir) channels. Kir4.1 channels may comprise either Kir4.1 homomers or Kir4.1/Kir5.1 heteromers [3]. Notably, Kir4.1 is expressed in rodents in the brain, ear, and kidney—the organs that are affected in humans with EAST/SeSAME syndrome [4–6].

The *KCNJ10* mutations associated with EAST/SeSAME syndrome cause amino acid substitutions or carboxyl-terminal deletions in Kir4.1 and, under experimental conditions, reduce the activity of Kir4.1 channels [1,2,7–12]. With the exception of A167V, all other identified *KCNJ10* mutations render Kir4.1 channels physiologically inactive, thereby reducing K^+ conductivity in the brain, ear, and kidney; this disruption of K^+ transport is likely responsible for the symptoms of EAST/SeSAME syndrome [4–6]. In contrast, the A167V mutant forms a functional channel under experimental conditions [7–9]. The homozygous A167V mutant shows approximately 60% residual activity, which is equivalent to the residual activity of the channels of the other heterozygous mutations associated with EAST/SeSAME syndrome. This suggested that reduced K^+ conductivity may not be solely pathogenic for the A167V mutation.

Recently, the A167V mutation was noted to render the Kir4.1/Kir5.1 heteromer nearly inactive in *Xenopus* oocytes [9]; this finding provided insights into the pathogenic role of the A167V mutation in the tubulopathy observed in EAST/SeSAME syndrome.

Abbreviations: Kir, inwardly rectifying K^+ channel; DCT, distal convoluted tubule; GFP, green fluorescent protein; HEK, human embryonic kidney; NCC, Na^+–Cl^- cotransporter; MAGI-1, membrane-associated guanylate kinase with inverted domain structure-1; MDCK, Madin-Darby canine kidney; PO, peroxidase

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The Kir4.1/Kir5.1 heteromer forms the predominant basolateral K^+ channel in the distal convoluted tubule (DCT) of rodent kidney [13]. This basolateral K^+ channel mediates recycling of the K^+ taken up by the basolaterally expressed Na^+/K^+ ATPase, and this recycling provides the driving force for Na^+ reabsorption at the DCT. Because K^+ recycling by the Kir4.1/Kir5.1 channel is indispensable to Na^+ reabsorption, inactivation of this channel by the A167V mutation causes renal salt wasting by reducing Na^+ reabsorption [14].

However, inactivation of the Kir4.1/Kir5.1 heteromer by the A167V mutation cannot explain the symptoms observed in the organs where Kir4.1 does not form heteromeric complexes with Kir5.1, such as the brain and ear [3,6]. In addition, the Kir4.1 (A167V)/Kir5.1 heteromer was found to retain its function in a mammalian cell line [7]. In contrast to the almost complete loss of function observed in *Xenopus* oocytes, the loss in COSm6 cells was moderate. Thus, activity of the Kir4.1(A167V) channels varies with the cell type: the channels are active in mammalian cells but are inactive in *Xenopus* oocytes. This finding suggests that Kir4.1 (A167V) may form active basolateral K^+ channels in tubular cells and that the finding in *Xenopus* oocytes cannot provide evidence for the pathogenesis of EAST/SeSAME syndrome.

We had previously reported that an anchor protein, membrane-associated guanylate kinase with inverted domain structure-1 (MAGI-1), facilitates the basolateral localization of Kir4.1 channels in tubular cells via direct interaction with Kir4.1 [15]. This finding suggested that interaction with anchor proteins is indispensable for functional expression of the Kir4.1 channels in several organs. In this study, we explored the role of intracellular localization of Kir4.1 channels in EAST/SeSAME syndrome-associated tubulopathy.

2. Materials and methods

2.1. Antibodies

Polyclonal anti-Kir5.1 and anti-MAGI-1 antibodies were raised and affinity-purified as described previously [3,15]. Polyclonal anti-Kir4.1 antibody (Sigma, Saint Louis, MO), anti-GFP antibodies (Clontech Laboratories, Palo Alto, CA), PO-conjugated IgGs (Pierce, Rockford, IL), Alexa Fluor 594 anti-rabbit IgG $(Fab')_2$ (Molecular Probes, Eugene, OR), and fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (Dako, Glostrup, Denmark) were purchased.

2.2. Immunohistochemistry

For immunohistochemical analysis of human kidney, renal biopsy specimens with minor abnormalities were used with the approval of the ethics committee of our institute. Frozen sections (1–2 μm) were incubated in blocking buffer (Dako), and then with the anti-Kir4.1 antibody followed by incubation with an excess (1:50 dilution) of Alexa Fluor 594 anti-rabbit IgG $(Fab')_2$ to saturate the antibody epitopes. After extensive washing, the sections were incubated with the anti-Kir5.1 antibody and FITC-labeled anti-rabbit IgG (1:200). Anti-Kir4.1 and anti-Kir5.1 antibodies were used at a final concentration of 2 μg/mL. Microscopy was performed using an Olympus model Bx50 (Olympus Medical Science, Tokyo, Japan).

2.3. Construction and transient expression of Kir channels

The coding sequences of human Kir4.1 and Kir5.1 (GenBank Accession Number NG009379, NG016411 and NM018658) were PCR-amplified from the human genome and inserted into mammalian cell expression vectors pcDNA3 (Invitrogen, Carlsbad, CA) and pEGFP-C1 (Clontech Laboratories), as described [16]. Point mutations were introduced with the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The expression vectors were transfected into Madin-Darby canine kidney (MDCK) II cells and human embryonic kidney (HEK) 293T cells with Lipofectamine 2000 (Invitrogen). Cytology and immunoblotting were performed 2–3 days after transfection.

2.4. Cytology

Cytological observation of MDCK II and HEK293T cells was performed as described previously [16,17]. MDCK II cells were cultured to confluence on polycarbonate Millicell transwell filters (Millipore, Bedford, MA) and expression of a tight junction protein, ZO-1, was observed on the top of the lateral wall. Confocal microscopy was performed using a model LSM5 PASCAL microscope (Carl Zeiss Co., Ltd.).

2.5. Immunoblotting and cell surface biotinylation

Immunoblotting, immunoprecipitation, and glutathione *S*-transferase (GST) pull-down assays were performed as described previously [3,18]. Cell surface biotinylation was performed as described previously [18]. To detect the blotted channels, anti-Kir4.1 (1 μg/mL), anti-Kir5.1 (0.8 μg/mL), or anti-GFP antibody (0.2 μg/mL) was used in blocking buffer [80 mM NaCl, 50 mM Tris–HCl (pH7.5), 4% (w/v) skim milk and 0.2% Triton X-100], and the immunoreacted bands were detected by SuperSignal West Dura (Pierce). Band intensity was quantified with NIH Image software (National Institutes of Health, Bethesda, MD). The degree of band intensity for mutant relative to that of the wild type was calculated as the intensity of mutant divided by that of the wild type.

2.6. Statistical analysis

The degree of band intensity was expressed as the mean ± standard deviation (S.D.). Comparisons were performed by the paired two-tailed Student's t test. Probability values of $P < 0.01$ were considered statistically significant.

3. Results

3.1. Composition of the basolateral K^+ channel in human DCT

We determined the composition of the basolateral K^+ channels in human renal tubules by the co-immunostaining method. For this, we used anti-Kir4.1 and anti-Kir5.1 antibodies; the specificities of these antibodies were confirmed by immunoblotting (Supplementary Fig. 1). Each antibody specifically recognized the corresponding human Kir channel tagged with green fluorescent protein (GFP), and none of the antibodies cross-reacted with other Kir channels. We also confirmed the saturation of the first antibody with Alexa Fluor 594 IgG $(Fab')_2$ by no spill-over staining using FITC without the second antibody (Supplementary Fig. 2). This analysis revealed localization of Kir5.1 to the Kir4.1-expressing tubules (Fig. 1). In these tubules, Kir5.1 and Kir4.1 showed basolateral distribution. Kir5.1 immunostaining was also observed in tubules not showing positive immunostaining for Kir4.1; in these tubules, Kir5.1 showed clustered distribution.

3.2. Kir4.1/Kir5.1 heteromer formation and role of MAGI-I in basolateral K^+ channel expression

Co-localization of Kir4.1 and Kir5.1 to the basolateral side suggests that the Kir4.1/Kir5.1 heteromer forms the predominant basolateral K^+ channel in human DCT, as it does in rodent DCT [13]. Channel assembly at the basolateral side also seems to