

Identification of human Kir2.2 (KCNJ12) gene encoding functional inward rectifier potassium channel in both mammalian cells and *Xenopus* oocytes

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Received 4 September 2002; revised 23 September 2002; accepted 27 September 2002

First published online 10 October 2002

Edited by Maurice Montal

Abstract Arginine residue at position 285 (R285) in the intracellular C-terminal domain of inward rectifier potassium channel Kir2.2 is conserved in many species, but missing in previously reported human Kir2.2 sequences. We here identified the human Kir2.2 gene in normal individuals, which contained R285 in the deduced amino-acid sequence (hKir2.2/R285). All 30 individuals we examined were homozygous for Kir2.2/R285 gene. The hKir2.2/R285 was electrophysiologically functional in both mammalian cells and *Xenopus* oocytes. However, the hKir2.2 missing R285 was functional only in *Xenopus* oocytes, but not in mammalian cells. Thus, R285 in Kir2.2 is important for its functional expression in mammalian cells.

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Key words: Potassium channel; Inward rectifier; Kir2.2; KCNJ12; Mammalian cell; *Xenopus* oocyte

1. Introduction

The strong inward rectifier potassium channels conduct currents at voltages around its reversal potential, but little currents at depolarized voltages. Due to this property, these channels stabilize the resting membrane potential, control the excitability of the cells, and repolarize the action potentials in many cell types including cardiac myocytes and neurons [1]. The members of the potassium channel gene subfamily Kir2 encode the pore-forming subunits of the strong inward rectifier potassium channels. The topology model of the subunits has two transmembrane segments (M1 and M2) with a potassium selective filter-forming P-loop in between, and cytoplasmic N- and C-terminal domains [2]. Kir2 channels are formed by co-assembly of four pore-forming subunits [3,4], and regions in the C-terminus determine their multimerization [5]. Among the genes in Kir2 family, Kir2.1 and Kir2.2 have attracted attention because the channels expressed from these clones show electrophysiological properties resembling the strong inward rectifier I_{K1} of cardiac ventricular myocytes [2,6,7]. Thus, the molecular basis of ion permeation, gating properties and the nature of inward rectification have

been studied using these channels. Indeed, a study conducted using the mice with a targeted deletion of Kir2.1 or Kir2.2 gene has recently suggested that Kir2.1 and Kir2.2 channels provide the major component of I_{K1} in the neonatal mouse heart [8].

We have cloned Kir2.2 (KCNJ12) gene from human genomic DNA from normal individuals. The deduced amino acid sequence had arginine residue at amino acid position 285 (R285) in the C-terminal domain. This arginine residue is not found in the previously reported human Kir2.2 (hKir2.2) obtained from an atrial cDNA library [9] and genomic libraries [10]², [11]. We examined the Kir2.2 coding sequence in normal Asians. Here we report that hKir2.2 containing R285 is common in the normal individuals. The arginine residue is conserved in the Kir2.2 of mouse [12], rat [13] and guinea-pig [14]. Kir2.1 also possess a positively charged amino acid residue, lysine, at position 284 (K284) in the highly conserved region [15]. We studied the role of R285 in Kir2.2 and K284 in Kir2.1, and showed that these residues are critical determinants for functional expression of Kir2 channels in mammalian cells.

2. Materials and methods

2.1. Molecular cloning and sequence analysis

Genomic DNA was extracted from peripheral blood from normal Asians using QIAamp DNA Blood Mini kits (Qiagen). The study was approved by the Nagasaki University Institutional Review Board. The complete coding regions of hKir2.1 and hKir2.2 genes were obtained using polymerase chain reaction (PCR), since these genes are intronless [13,16]. Sequences of forward and reverse primers for hKir2.1 were 5'-AGC AGA AGC ACT GGA GTC CCC AGC AGA AGC-3' and 5'-AGG AAT CAG TCA GTC ATA TCT CCG A-3', respectively. Sequences of forward and reverse primers for hKir2.2 were 5'-CAC GAG GCC TGG AGC TAG CCT GGG GGC GAG-3' and 5'-TGT CGG CCA AGG TTG GCT CAG ATC TCT GAC-3', respectively. Amplifications were performed with 94°C denaturation (30 s), 58°C annealing (30 s) and 72°C elongation (90 s) for 30 cycles. The resulting fragments were subcloned into pCR2.1 (Invitrogen) and sequenced on both strands using an ABI 377 or 310 automated DNA sequencer (Perkin-Elmer). The PCR amplification for hKir2.1 generated a fragment (1328 bp) that contained the entire coding region of hKir2.1 gene [15].

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² Personal communication: Dr. N. Inagaki (Akita University School of Medicine) confirmed the sequence of the hKir2.2 missing R285 and has no data on the expression of the clone in mammalian cells.

2.2. Site-directed mutagenesis

Mutations of hKir2.1, hKir2.2 and mouse Kir2.2 (mKir2.2) [12] (cloned in pCDNA3) were generated by oligonucleotide-mediated site-directed mutagenesis using QuickChange Site-Directed Mutagenesis kits (Stratagene). The mutagenesis primers were; hKir2.1/ΔK284: 5'-TTA TAT GAT TTG AGT CAG GAC ATT GAC AAC-3', hKir2.2/ΔR285: 5'-GTC CTT CGG CAT CAG CCA GGA CCT GGA GAC-3', mKir2.2/ΔR285: 5'-ACT GTT TGG CAT TAG CCA GGA CCT TGA GAC-3', mKir2.2/R285D: 5'-ACT GTT TGG CAT TAG CGA TCA GGA CCT TGA GAC-3', mKir2.2/R285L: 5'-ACT GTT TGG CAT TAG CCT CCA GGA CCT TGA GAC-3'. The entire coding regions of mutant clones were sequenced on both strands.

2.3. Functional expression of Kir genes in mammalian cells and *Xenopus* oocytes

Kir genes were inserted into the mammalian expression vector pCXN2 [17]. L cells (mouse fibroblast cell line) and 293T cells (human kidney cell line) were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 5% (L cells) or 10% (293T cells) heat-inactivated fetal bovine serum and penicillin (200 U/ml)–streptomycin (200 μg/ml). Cells were plated at 1.5×10^5 cells/35 mm dish 1 day prior to transfection. They were transfected with 0.4 μg of plasmid DNA containing one of the Kir genes and 0.04 μg of the plasmid DNA encoding the enhanced green fluorescence protein (pEGFP-N1, Clontech) using an Effectene transfection reagent (Qiagen). At 24–56 h after transfection, the cells expressing exogenous genes were identified by visualizing the fluorescence lights with an inverted fluorescence microscope, and whole-cell currents were recorded by the conventional patch-clamp technique using a patch-clamp amplifier Axopatch 200B (Axon Instruments) [18]. The bath solution contained (in mM): NaCl 140, KCl 5.4, MgCl₂ 0.5, CaCl₂ 1.8, NaH₂PO₄ 0.33, glucose 5.5 and HEPES 5, pH 7.4. The pipette solution contained (in mM): KCl 30, K aspartate 85, KH₂PO₄ 10, K₂EDTA 2, K₂ATP 2, MgCl₂ 5, and HEPES 5, pH 7.2.

Experimental procedures used for functional expression in *Xenopus* oocytes were similar to those described elsewhere [19]. Capped cRNAs for Kir2 were synthesized in vitro with T7 RNA polymerase using MEGAscript[®] kits (Ambion). Approximately 10 ng of cRNA was injected in each oocyte. Electrophysiological measurements were made between 3 and 5 days after the injection using a two-electrode clamp amplifier TEV-200 (Dagan). The bath solution contained (in mM): KCl 96, NaCl 2, CaCl₂ 1.8, MgCl₂ 1, and HEPES 5, pH 7.4.

For voltage stimulations and data acquisitions we used pCLAMP software (Axon Instrument). Comparisons were made using an unpaired Student's *t*-test. Results were expressed as the mean ± S.E.M. Data were recorded at a room temperature (23–25°C).

2.4. Confocal laser scanning of EGFP-fused Kir channels

C-terminal fusion constructs of Kir channel subunits with EGFP were generated as follows. To remove the stop codon and to introduce a novel *Bam*HI site at the 3'-end of hKir2.2 and hKir2.1 genes, entire coding regions of the genes in pCR2.1 were amplified by PCR using the following primers. For hKir2.2, the forward primer was 5'-CAC GAG GCC TGG AGC TAG CCT GGG GGC GAG-3', and the *Bam*HI reverse primer 5'-ATG GGA TCC CAG ATC TCT GAC TCC-3'. For hKir2.1, the forward primer was 5'-AGC AGA AGC ACT GGA GTC CCC AGC AGA AGC-3', and the *Bam*HI reverse primer 5'-ATG GGA TCC CAT ATC TCC GAC TCT-3'. The resulting PCR products were subcloned into pCR-Blunt II-TOPO vector (Invitrogen), and were verified by sequencing on both strands. The *Eco*RI/*Bam*HI fragments from the resulting constructs were subcloned into EGFP expression plasmid pEGFP-N1 to obtain EGFP fusions with the C-terminus of Kir proteins. To generate EGFP-fused constructs of mKir2.2, *Hind*III/*Psh*AI fragments of the genes in pCDNA3 were inserted into pEGFP-N1, resulting in in-frame EGFP fusions with the C-terminus of mKir2.2 proteins. The *Psh*AI digestion truncated 15 nucleotides of the 3'-end of mKir2.2, including stop codon.

The generated plasmids, hKir2.2/R285-EGFP, hKir2.2/ΔR285-EGFP, hKir2.1-EGFP, hKir2.1/ΔK284-EGFP, mKir2.2-EGFP, and mKir2.2/ΔR285-EGFP, were transfected to 293T cells. At 36 h after transfection, cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 30 min, then with 5% sucrose in PBS for 10 min, and finally with 40% glycerol in PBS at room temperature. The GFP fluorescence was visualized using an

invert laser-scanning microscope Zeiss LSM 410. The GFP fluorescence was excited using a 488-nm laser and detected using a 515–540-nm band pass filter.

2.5. Western blot analysis

Kir genes in pCXN2 or pEGFP-N1 were transiently transfected into 293T cells. At 36 h after transfection, cells in 35 mm plates were rinsed with PBS, scraped off into 400 μl lysis solution containing (in mM): NaCl 150, Tris-HCl 20, and 1% NP40, supplemented with a protease and phosphatase inhibitor cocktail Complete (Boehringer-Mannheim), and homogenized using a sonicator. After 1 h incubation on ice, insoluble debris was removed by centrifugation at $13000 \times g$ for 7 min. The lysates (30 μg) were boiled in sample buffer (2% SDS, 2% mercaptoethanol, 20% glycerol, and 0.0015% bromophenol blue in Tris-HCl 120 mM, pH 6.8) for 3 min, separated on 10% SDS-polyacrylamide gels, and then blotted electrophoretically onto nitrocellulose membranes (Bio-Rad). After blotting, the membranes were blocked for 1 h with 4% bovine albumin (Sigma, fraction V) and 0.1% Tween 20 in Tris-buffered saline, and were incubated for 2 h at room temperature with polyclonal antibodies against Kir2.1 (Alomone Labs) at 1:500 dilution or GFP (Clontech) at 1:100 dilution. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody, and the signal was detected using ECL detection kits (Amersham) and an ECL mini-camera (Amersham).

3. Results and discussion

The PCR products obtained using the primers for hKir2.2 contained two different products (1368 bp and 1371 bp). DNA sequencing revealed that the fragment (1368 bp) included the entire coding region of Kir2.2v gene (KCNJN1) [10]. The Kir2.2v, which does not express any currents, has R285 and differs in 21 amino acids from hKir2.2. The fragment (1371 bp) included the complete coding region of hKir2.2 gene (KCNJ12) with three additional nucleotides CGG at nucleotides 853–855 in the open reading frame, encoding arginine at amino acid position 285, compared with the previously reported hKir2.2 gene [9–11]. All the three previously reported hKir2.2 genes were missing R285. To clarify the difference, we designate our cloned hKir2.2 (accession number: AB074970) as hKir2.2/R285 and the previously reported ones as hKir2.2/ΔR285. To examine the ratio of

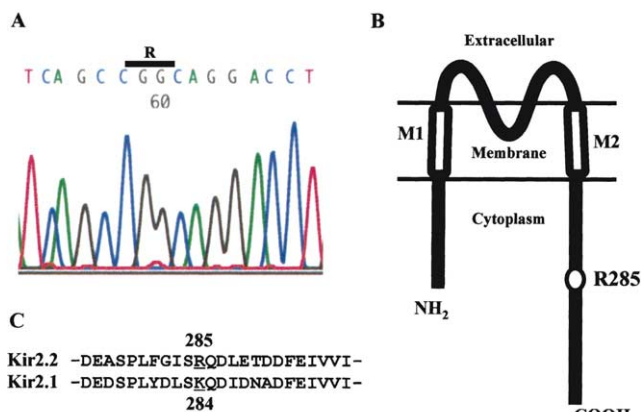


Fig. 1. A: Direct sequence of PCR fragments generated from genomic DNA. Shown is a representative chromatogram from an individual homozygous for hKir2.2/R285. Sequences of the primer for the sequencing reactions were 5'-GGCCTGGACCGCATCTTCT-3'. The CGG indicated by the line corresponds to arginine (R) residue. B: Putative membrane topology of Kir channel subunit. R285 is located in the cytoplasmic domain after the second transmembrane region. C: Amino-acid sequences of the regions surrounding R285 of Kir2.2 and K284 of Kir2.1.

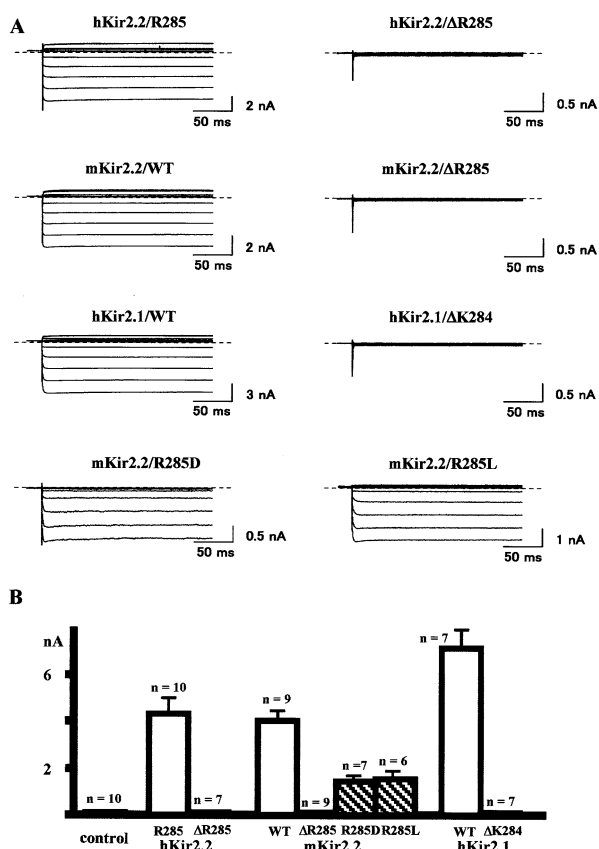


Fig. 2. A: Representative current traces recorded from 293T cells transfected with hKir2.2/R285, hKir2.2/ Δ R285, mKir2.2/WT, mKir2.2/ Δ R285, hKir2.1/WT, hKir2.1/ Δ K284, mKir2.2/R285D, and mKir2.2/R285L, as indicated. Currents were recorded from a holding potential of -30 mV stepped from -120 mV to -20 mV in 10 mV increments. Bar graph (B) compares the magnitude of the inward currents recorded at -120 mV in 293T cells (control) and 293T cells expressing Kir channels, as indicated.

hKir2.2/R285 and hKir2.2/ Δ R285 in normal populations, we examined the hKir2.2 gene by sequencing directly the genomic DNAs from 30 normal Asians. All the 30 individuals examined were homozygous for hKir2.2/R285 gene (Fig. 1A). Insertion of the three nucleotides, CGG, creates an *Ngo*MIV restriction endonuclease site at nucleotide 851 in the open reading frame. The direct sequencing results were thus confirmed using the *Ngo*MIV digestion of the PCR fragment (data not shown). Wible et al. [9] cloned hKir2.2/ Δ R285 using reverse-transcriptase (RT)-PCR from human heart. It is possible that in case of RT-PCR the lack of three nucleotides, CGG, was caused by the reverse transcription step. Namba et al. [10] screened a human genomic library and isolated hKir2.2/ Δ R285 gene. Hugot et al. [11] directly sequenced a human genomic library and identified hKir2.2/ Δ R285 gene. In these two studies, the lack of three nucleotides is not due to any cloning artifacts, since they obtained the hKir2.2/ Δ R285 clones directly from genomic libraries. The sequence data missing R285 [10] was definitely confirmed by Dr. Inagaki (personal communication). These indicate that some populations carry hKir2.2/ Δ R285 gene.

The additional arginine residue in hKir2.2/R285 is located in the intracellular C-terminal domain (Fig. 1B). To examine whether the channel proteins with and without this residue show any functional differences, site-directed mutagenesis

was carried out to delete R285 in Kir2.2/R285. Surprisingly, 293T cells transfected with hKir2.2/ Δ R285 produced no measurable potassium currents, while those transfected with hKir2.2/R285 expressed prominent inward rectifier potassium currents (Fig. 2A). Current amplitudes measured at the end of the pulse at -120 mV were -4.28 ± 0.68 nA, -0.07 ± 0.01 nA, and -0.07 ± 0.01 nA for hKir2.2/R285, hKir2.2/ Δ R285, and untransfected 293T cells, respectively. In L cells, transfection with hKir2.2/R285 also produced inward rectifier potassium currents of significant amplitudes, whereas that with hKir2.2/ Δ R285 produced no such currents (data not shown). To confirm the importance of the arginine residue, we also deleted R285 of mKir2.2 and the positively charged residue lysine at the corresponding position 284 of hKir2.1 (Fig. 1C). 293T cells transfected with mKir2.2/ Δ R285 or hKir2.1/ Δ K284 did not express significant potassium currents, while those transfected with the wild-type channels (mKir2.2/WT or hKir2.1/WT) expressed large inward rectifier potassium currents (Fig. 2B,C). The current amplitudes at -120 mV were -3.99 ± 0.48 nA, -0.07 ± 0.01 nA, -7.11 ± 0.81 nA, and -0.09 ± 0.02 nA for mKir2.2/WT, mKir2.2/ Δ R285, hKir2.1/WT, and hKir2.1/ Δ K284, respectively. We thus concluded that R285 of Kir2.2 and K284 of Kir2.1 are critical for the functional expression in mammalian cells.

To examine whether the positive charge of the arginine residue is important, we constructed differently charged mutants using mKir2.2 (mKir2.2/R285D and mKir2.2/R285L). As shown in Fig. 2, their amplitudes were markedly smaller than those of mKir2.2/WT ($P < 0.002$). The current amplitudes measured at -120 mV were -1.41 ± 0.27 nA and -1.50 ± 0.30 nA for mKir2.2/R285D and mKir2.2/R285L, re-

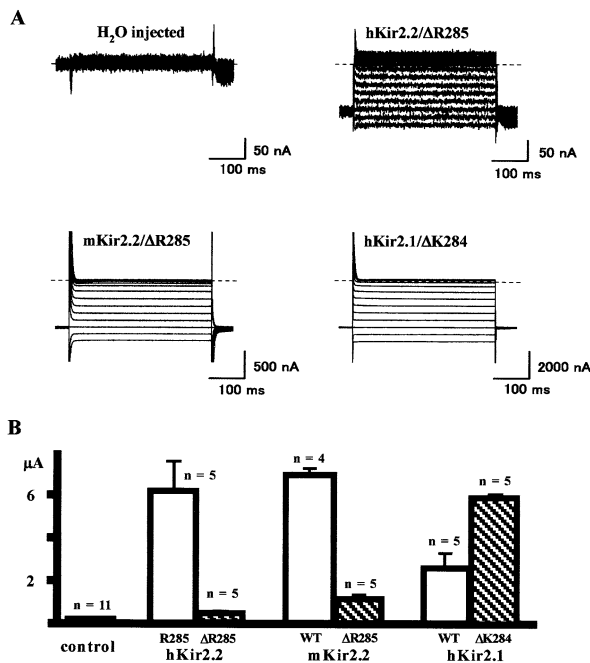


Fig. 3. A: Representative Ba²⁺ sensitive (500 μ M BaCl₂) current traces recorded from *Xenopus* oocytes injected with H₂O or cRNAs for hKir2.2/ Δ R285, mKir2.2/ Δ R285, and hKir2.1/ Δ K284, as indicated. Currents were recorded from a holding potential of -110 mV stepped from -130 mV to 10 mV in 10 mV increments. Bar graph (B) compares the magnitude of the currents recorded at -130 mV in *Xenopus* oocytes injected with H₂O (control) or with cRNAs for Kir channels, as indicated.

spectively. A conserved positively charged mutant, mKir2.2/R285K, produced inward rectifier potassium currents comparable to those seen with the wild-type mKir2.2 (data not shown). These results suggested that the positively charged residue is important for the functional expression of Kir2.2.

In contrast to the findings with mammalian cells, expression of hKir2.2/ Δ R285, mKir2.2/ Δ R285 and hKir2.1/ Δ K284 in *Xenopus* oocytes produced inward rectifier potassium currents (Fig. 3). This agrees with the previous reports demonstrating the functional expression of hKir2.2/ Δ R285 only in *Xenopus* oocytes [10,11]. The current amplitudes at -130 mV were -0.13 ± 0.01 μ A, -0.31 ± 0.04 μ A, -1.10 ± 0.29 μ A, and -5.87 ± 0.14 μ A in the oocytes injected with H₂O, hKir2.2/ Δ R285, mKir2.2/ Δ R285, and hKir2.1/ Δ K284, respectively. Different results between mammalian cells and *Xenopus* oocytes have been also noted for mutants of the human heart sodium channel gene, SCN5A [20], and the cystic fibrosis transmembrane conductance regulator (CFTR) [21,22,23]. Although the underlying mechanism of the different results remains to be unknown, we should be cautious in using *Xenopus* oocytes as a heterologous expression system to study channel functions.

To gain an insight into the mechanism of the function loss of the Kir channels, we examine the cellular localization of the Kir channels by constructing cDNA encoding EGFP-fused Kir channel subunits. Laser confocal microscopy demonstrated that the EGFP derived autofluorescence is observed in both the peripheral region and the cytoplasm of the cells expressing hKir2.2/R285-EGFP, mKir2.2-EGFP and hKir2.1-EGFP (Fig. 4). In contrast, the autofluorescence was mainly observed in the cytoplasm of the cells expressing EGFP-fused hKir2.2/ Δ R285, mKir2.2/ Δ R285 and hKir2.1/ Δ K284. These

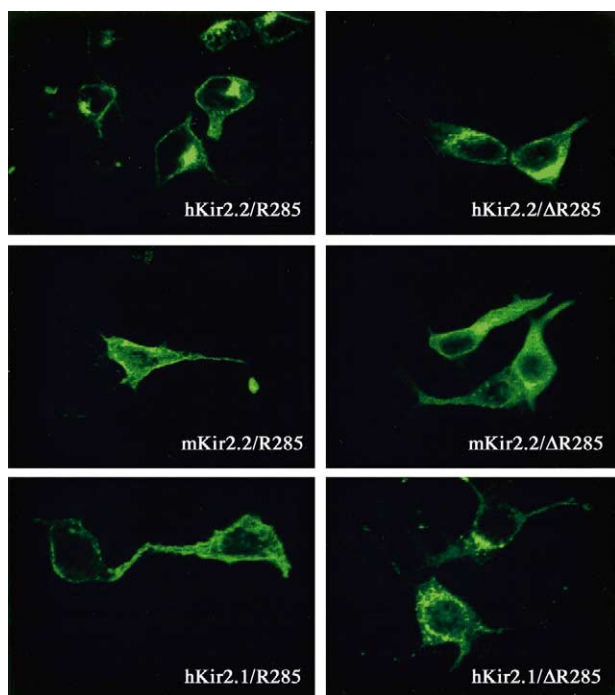


Fig. 4. Confocal microscopic analysis of the distribution of Kir2.1 and Kir2.2 channel proteins. Shown are the representative confocal images of 293T cells expressing EGFP-fused Kir channel proteins, as indicated. The subcellular localization of the GFP autofluorescence was examined at 36 h after transfection.

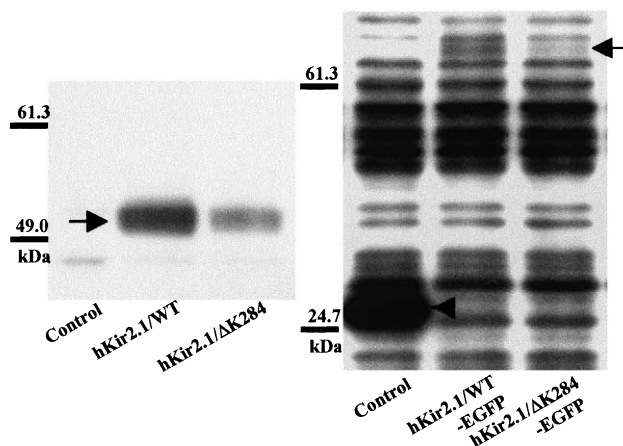


Fig. 5. Western blot analysis of hKir2.1 channel subunits and EGFP-fused hKir2.1 channel subunits. Both hKir2.1/ Δ K284 and hKir2.1/ Δ K284-EGF were synthesized as full length proteins. A: Arrow indicates hKir2.1 subunits. Control represents 293T cells transfected with pCXN2. B: Arrow indicates EGFP-fused hKir2.1 subunits. Arrow head indicates EGFP protein. Control represents 293T cells transfected with pEGFP-N1.

observations indicate that the cell-surface localization of hKir2.2/ Δ R285-EGFP, mKir2.2/ Δ R285-EGFP and hKir2.1/ Δ K284-EGFP is markedly decreased. We asked if the inactive channel subunits were synthesized as full-length proteins. Using antibodies raised against rat Kir2.2 [4], we tried to analyze hKir2.2 and mKir2.2 proteins. However, the antibodies were not reactive with these channel proteins (data not shown). Anti-Kir2.1 antibodies recognized both hKir2.1/WT and hKir2.1/ Δ K284 proteins at approximately 50 kDa (Fig. 5A), indicating that the inactive channels were synthesized as full-length proteins. The anti-GFP antibodies recognized both EGFP-fused hKir2.1/WT and EGFP-fused hKir2.1/ Δ K284 proteins as a band of ~ 77 kDa (Fig. 5B). The molecular mass of 77 kDa is in good agreement with the predicted molecular mass of EGFP-fused hKir2.1 (~ 50 kDa for hKir2.1 and ~ 27 kDa for EGFP). The expression levels of the subunits were somewhat lower in the deletion mutant than in the wild type (Fig. 5A,B). Previous studies concerning a chloride channel have shown that a deletion mutant of CFTR is degraded rapidly and fails to reach the plasma membrane [24]. We thus consider that R285 in Kir2.2 and K284 in Kir2.1 might be implicated to play an important role in the forward trafficking of Kir channel proteins as in the case of CFTR. The C-terminal domain including K284 is reported to be responsible for the multimerization of Kir2.1 channel subunits [5]. Therefore, the positively charged amino acid might contribute to the multimerization of Kir channel subunits, or the stable localization of the channels in the plasma membrane.

In summary, the finding that all individuals we examined were homozygous for hKir2.2/R285 suggested that the Kir2.2 gene containing R285 is common in Asians, although the previously reported hKir2.2 missing R285. We demonstrated that the absence of R285 in Kir2.2 impairs the cell surface localization of the Kir channels in mammalian cells.

Acknowledgements: The authors thank Dr. Y. Kurachi for the mouse Kir2.2 clone, and Dr. C.A. Vandenberg for antibodies against rat Kir2.2. This study was supported by a grant from the Ministry of Education, Science, Technology, Sports, and Culture, Japan.

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