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# Molecular cloning and characterization of Kv6.3, a novel modulatory subunit for voltage-gated $K^+$ channel Kv2.1[1]

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**Abstract We report identification and characterization of Kv6.3, a novel member of the voltage-gated $K^+$ channel. Reverse transcriptase-polymerase chain reaction analysis indicated that Kv6.3 was highly expressed in the brain. Electrophysiological studies indicated that homomultimeric Kv6.3 did not yield a functional voltage-gated ion channel. When Kv6.3 and Kv2.1 were co-expressed, the heteromultimeric channels displayed the decreased rate of deactivation compared to the homomultimeric Kv2.1 channels. Immunoprecipitation studies indicated that Kv6.3 bound with Kv2.1 in co-transfected cells. These results indicate that Kv6.3 is a novel member of the voltage-gated $K^+$ channel which functions as a modulatory subunit.** 

*Key words:* Voltage-gated $K^+$ channel; Kv6.3; Kv2.1; Modulatory subunit

## 1. Introduction

Voltage-gated $K^+$ channels form the largest and most varied family of ion channels (Kv channel family). These channels play an essential role in controlling cellular excitability in the nervous system and regulate a variety of neuronal properties, such as the interspike membrane potential, action potential waveform and firing frequency [1]. These findings indicate the importance of voltage-gated $K^+$ channels in neuronal signal transduction and processing. Their contribution to behavior phenotype, such as learning and memory, has also been studied [2,3].

To date, many members of this Kv channel family have been identified and characterized. They have six transmembrane domains (TM1–6) and one pore-forming domain, which is essential for permeation of $K^+$, between TM5 and TM6 [4–7]. This secondary structure is evolutionarily conserved. On the basis of functional characterization of members of the voltage-gated $K^+$ channel family, these members have been divided into two different subunits, $\alpha$ and $\gamma$ subunits [8]. Although these subunits have a similar secondary structure and sequence similarity, $\alpha$ subunits show voltage-gated channel activity by themselves, whereas $\gamma$ subunits do not. However, when $\gamma$ subunits are co-expressed with $\alpha$ subunits, they modify the voltage-gated channel activity which is generated by $\alpha$ subunits [9–13]. These results demonstrate that $\gamma$ subunits function as a modifier of $\alpha$ subunits, and this modification may express the original activity in living cells.

We attempted to identify a novel member of the Kv channel family from the human genomic sequencing database. A novel gene encoding voltage-gated $K^+$ channel subunits was isolated which has the most similarity to the Kv6.2 channel and is mainly expressed in the brain. In this study we report the molecular cloning, tissue distribution, channel activity, and binding to the Kv2.1 channel of this gene, Kv6.3. In addition, the identification and distribution of a rat ortholog of this gene are described.

## 2. Materials and methods

### 2.1. Molecular cloning of human Kv6.3

The human Kv6.2 amino acid sequence was used for a TBLASTN search against the human draft genome sequencing database at the National Center for Biotechnology Information (NCBI). The genomic clone (GenBank accession number AC025125) was identified. To identify the 5′- and 3′-ends of the cDNA corresponding to this fragment, 5′- and 3′-rapid amplification of cDNA ends (RACE) was performed using the Human Brain Marathon-Ready cDNA (Clontech, Palo Alto, CA, USA) and primers derived from the genomic fragment. Amplified fragments were directly cloned into the plasmid pCR-TOPO vector (Invitrogen, Carlsberg, CA, USA) and sequenced on both strands. The determined sequences were assembled into one contig with an open reading frame encoding 437 amino acids, which is called human Kv6.3. Finally, to verify that human Kv6.3 cDNA including the entire open reading frame could be cloned from an independent source, human brain poly(A)$^+$ RNA (Clontech) was used for the reverse transcriptase-polymerase chain reaction (RT-PCR). The RT reaction was primed with random hexamer. The primers for PCR were 5′-AGGTGTGCGACGACTACGA-3′ and 5′-GATGATGACAATGCCACTGC-3′. The amplified fragment was subcloned into pCR-TOPO and sequenced on both strands.

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[1] The human and rat nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers AB070604 and AB070605, respectively.

*Abbreviations:* PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; CDS, coding sequence

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*2.2. Molecular cloning and isolation of rat Kv6.3*

To isolate a rat homolog cDNA of Kv6.3, a TBLASTN search of the expressed sequence tag (EST) database of GenBank, using the human Kv6.3 amino acid sequence, retrieved a rat sequence with the accession number AI043703 (523 bases). This sequence corresponded to amino acid residues 200–383 of human Kv6.3 and showed high similarity (93%). To identify the 5′- and 3′-ends of the cDNA corresponding to the EST, 5′- and 3′-RACE were performed using the Rat Brain Marathon-Ready cDNA (Clontech) and primers derived from the EST sequence. The amplified fragments were directly cloned into the plasmid pCR-TOPO and sequenced on both strands. The determined sequences were assembled into one contig with an open reading frame encoding 345 amino acids. This sequence is externally similar (95%) to human Kv6.3, indicating that this is the rat counterpart of Kv6.3.

*2.3. RT-PCR analysis*

Multiple human and rat poly(A)$^+$ RNAs (Clontech) were reverse-transcribed to generate first-strand cDNAs with Advantage RT-for-PCR Kit (Clontech). PCR was carried out with these first-strand cDNAs, corresponding to 5 ng poly(A)$^+$ RNA. PCR primers were designed from the human Kv6.3 cDNA sequence: 5′-TGTCAAGAGACCCCTGAA-3′, 5′-TCCATAGCCAACTGTAGTCA-3′ and from the rat Kv6.3 cDNA sequence: 5′-GCATCGTGCGGTTCATCGTC-3′, 5′-CATGTCTCCATAGCCCACTG-3′. PCR was performed for 35 cycles at 98°C for 15 s and 70°C for 1 min. The amplified fragments were subcloned into pCR-TOPO vector and sequenced on both strands to confirm the PCR products as human or rat Kv6.3. As a control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using specific primers (Clontech).

*2.4. Electrophysiological studies*

Previously isolated cDNA of human Kv6.3 was subcloned into pcDNA3.1 expression vector (Invitrogen). L929 cells and L929 cells stably expressing human Kv2.1 were co-transfected with the vector and green fluorescent protein expression vector using Lipofectamine (Invitrogen), as described previously [14]. Electrophysiological studies were carried out 2–4 days after transfection.

Voltage clamp measurements were performed as described previously [15]. Currents were measured using whole-cell patch-clamp configurations with an Axopatch 200B patch-clamp amplifier using pClamp8 software (Axon Instruments). The external solution contained (in mM): KCl 145.4, $CaCl_2$ 2, HEPES 10, glucose 15, pH 7.4 with KOH. Patch pipettes (2–5 MΩ) were filled with the internal solution containing (in mM): KCl 125, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, HEPES 10, pH 7.2 with KOH. The activation characteristics of the Kv channels were determined from tail currents arising from channel deactivation on repolarizing to −84 mV from step membrane potentials between −64 and +36 mV. The tail current amplitudes were measured isochronally 5 ms after step pulses and normalized to the current obtained following a step to +36 mV. These data were fit to a modified Boltzmann equation:

$$I_{tail}/I_{max} = 1/(1 + \exp((V_{1/2} - V_m)/k))$$

where $I_{tail}$ is the current amplitude at membrane potential $V_m$, $I_{max}$ is the current amplitude used for normalization, $V_{1/2}$ is the half-activation potential, and $k$ is a slope factor [16]. Series resistance was compensated for at least 80%. All recordings were performed at room temperature (25°C).

*2.5. Immunoprecipitation of Kv6.3 with Kv2.1*

Tagged human Kv6.3 and human Kv2.1 were co-transfected into COS7 cells. 2 days after the transfection the cells were homogenized at 4°C for 1 h in a solubilizing buffer containing 1% Triton X-100, complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and phosphate-buffered saline (PBS). Insoluble material was removed by centrifugation at 15 000 × $g$ for 10 min at 4°C. Mouse anti-FlagM2 monoclonal antibody (Sigma-Aldrich) was added at 10 μg/ml for 2 h at 4°C by addition of protein G immobilized on agarose beads (Roche Diagnostics) with slow rocking. Pellets were washed three times in solubilizing buffer. The immunoprecipitated proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech UK, Buckinghamshire, UK). Blots were saturated with Block Ace (Dainihon Seiyaku Co., Osaka, Japan) for 1 h at room temperature and then incubated for 1 h with primary antibodies: anti-FlagM2 and anti-Kv2.1 (Upstate Biotechnology, Lake Placid, NY, USA). After several washes with PBS containing 0.05% Tween 20, the blots were revealed with purified goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:10 000) (Amersham Pharmacia Biotech UK) for 1 h at room temperature followed by incubation with substrate for enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech UK).

## 3. Results and discussion

*3.1. Molecular cloning of a novel putative voltage-gated $K^+$ channel, Kv6.3*

We identified a genomic clone, AC025750, which encoded a putative amino acid sequence that was partially similar to human Kv6.2, by searching the human draft genome sequencing database. 5′- and 3′-RACE studies using primers designed from this genomic clone caused an identification of the full-length coding sequence (CDS) (1314 bases). This CDS has an open reading frame encoding 437 amino acids with a predicted molecular mass of 49 kDa (Fig. 1A). Hydrophilicity analysis of the amino acid sequence showed that the novel clone belonged to the $K^+$ channel family with one pore-forming domain and six transmembrane domains (TM) (Fig. 1B). Since this novel clone is included in human genomic clone AC025750, which consists of 176 867 bases and is localized in chromosome 2 of the human genome, we performed sequence alignment with the CDS and AC025750. The alignment revealed that the CDS consisted of two exons and one intron spanning 49 198 bases.

A search of the GenBank database using the BLAST sequence alignment program revealed that the full amino acid sequence of the novel clone showed the closest similarity to human Kv6.2, which is a member of the voltage-gated $K^+$ channel family, and the expected value and identity are $6e^{-13}$ and 34%, respectively. Alignment of the amino acid se-

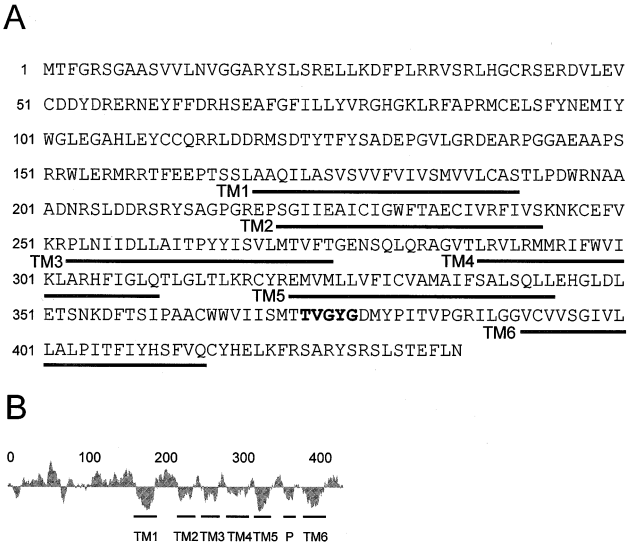


Fig. 1. A: Deduced amino acid sequence of hKv6.3. The six putative transmembrane regions (TM1–6) are underlined. Consensus sequences (TxGYG) within the $K^+$ channels are in boldface. B: Hydrophilicity plot of hKv6.3. The six putative transmembrane regions (TM1–6) and pour region (P) are underlined.

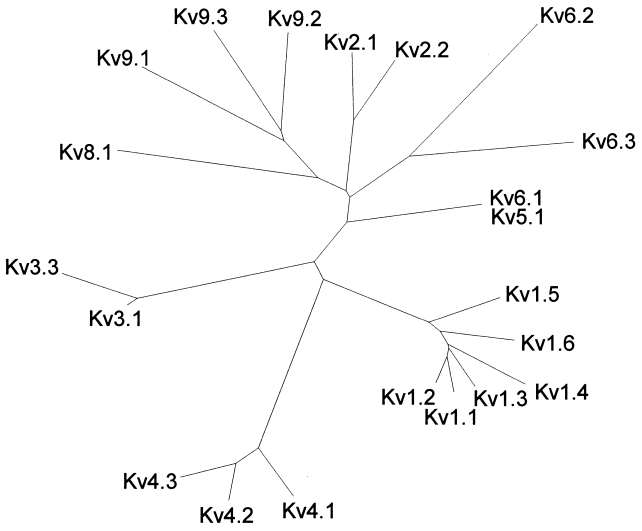


Fig. 2. Phylogenetic tree of the human Kv channel family, including hKv6.3. The tree was generated using the neighbor-joining algorithm of the Phylip program, on the basis of multiple alignment of conserved sequences of the pore domain analyzed with a Clustal W program.

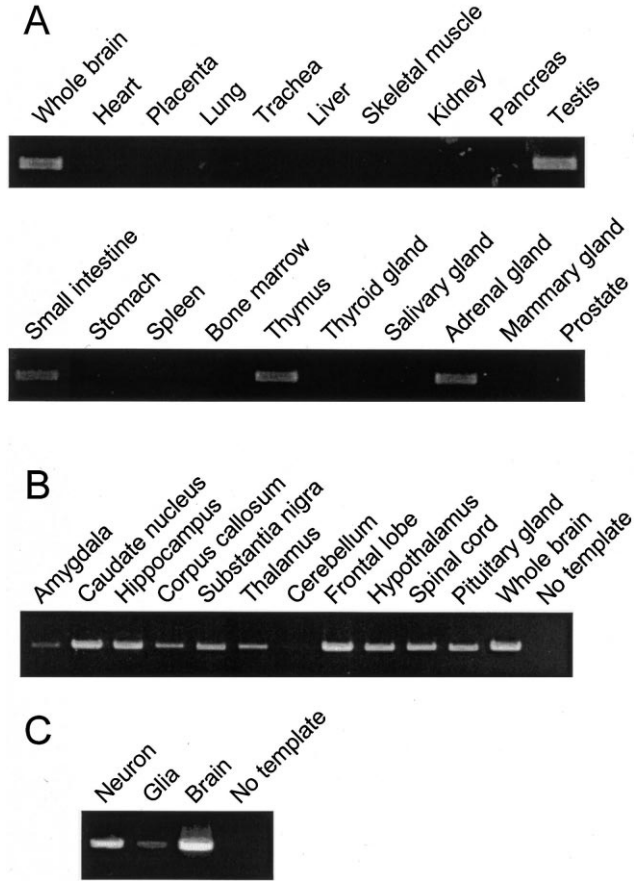


Fig. 3. Tissue distribution of Kv6.3. A: RT-PCR analysis of hKv6.3. A, B: Poly(A)$^+$ RNA (5 ng) from multiple human tissues and brain regions were reverse-transcribed with a random primer and amplified by PCR for hKv6.3. C: Total RNA from primary cultures of neurons or glial cells from the rat cortex were used for RT-PCR of rKv6.3. As the positive control of these RT-PCR reactions, GAPDH was amplified (data not shown).

quence of the novel clone with other voltage-gated $K^+$ channels showed low homology: 13.5–19.7%. Also, phylogenetic analysis suggested that this clone had a common ancestor with these $K^+$ channels and represents an additional branch in the Kv6 subfamily (Fig. 2). These results thus indicated that this novel clone was a new member of the voltage-gated $K^+$ channel, and moreover, was included in the Kv6 subfamily. Therefore, we named this novel clone human Kv6.3 (hKv6.3). Additionally, a rat ortholog of the Kv6.3 gene (rKv6.3) was identified from rat brain mRNA, which encodes 345 amino acids with 95.1% identity to hKv6.3.

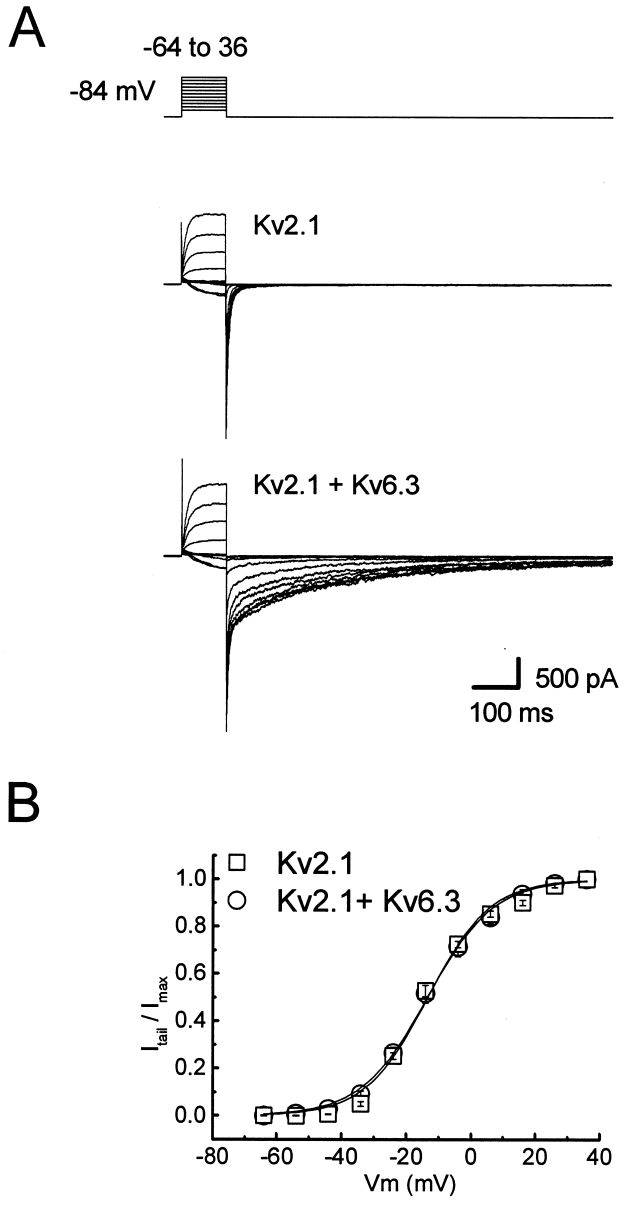


Fig. 4. Electrophysiological study of hKv6.3. A: Whole-cell macroscopic currents elicited in hKv2.1 stably expressed L929 cells and the cells transfected with hKv6.3. The holding potential was −84 mV, and the cells were depolarized from −64 to +36 mV by 10 mV increments. B: Voltage-dependent activation curves of hKv2.1 and hKv2.1/hKv6.3 currents. The normalized tail current amplitude ($I_{tail}$/$I_{max}$) was fit to a modified Boltzmann equation (see Section 2). Each data point is the mean ± S.E.M. ($n = 7$).

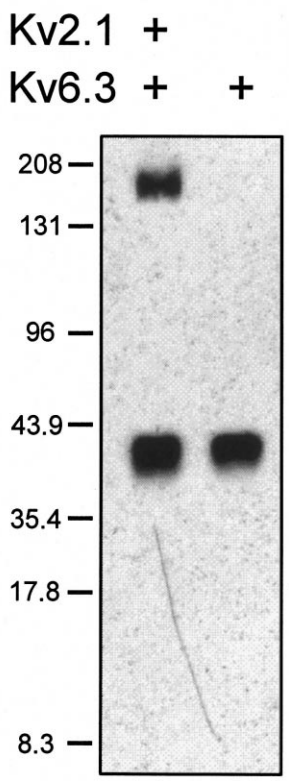


Fig. 5. Association of hKv6.3 with hKv2.1 in co-transfected COS7 cells. The possibility of physical association between hKv6.3 and hKv2.1 proteins was examined by co-immunoprecipitation reactions from co-transfected COS7 cells. Immunoprecipitation was performed by using anti-FLAG-tag antibodies (anti-hKv6.3), and co-precipitated hKv2.1 was detected with anti-hKv2.1 antibodies. The numbers on the left refer to the mobility of prestained molecular weight standards.

### 3.2. Tissue distribution of Kv6.3 mRNA

RT-PCR analysis for human multiple mRNA showed that hKv6.3 mRNA was highly expressed in the brain (Fig. 3A). Additional analysis revealed that hKv6.3 mRNA was widely expressed in various regions of the human brain (Fig. 3B).

Since hKv6.3 is expressed in the brain, we verified whether Kv6.3 was expressed in neuronal or glial cells of the brain. RT-PCR analysis with total RNA from neuronal and glial primary cultured cells from the rat cortex revealed that rKv6.3 was specifically expressed in neuronal cells (Fig. 3C). This selective expression is similar to that of Kv2.1, which was reported to be mainly expressed and function in the brain [17].

### 3.3. Electrophysiological study of hKv6.3

L929 cells transiently expressing hKv6.3 alone did not yield functional voltage-gated ion channels (data not shown). However, co-expression of hKv6.3 and human Kv2.1 (hKv2.1) gave rise to Kv channels mediating currents distinct from those of homomultimeric Kv2.1 channels (Fig. 4A). The deactivation kinetics of the heteromultimeric channels were obviously slower than those of the homomultimeric channels. Detailed analysis of the tail current amplitude revealed that the half-maximal activation potential and slope factor of the heteromultimeric channels ($V_{1/2} = -13.3 \pm 1.8$ mV; $k = 9.8 \pm 0.4$ mV; mean ± S.E.M.; $n = 7$) were similar to those of the homomultimeric channels ($V_{1/2} = -13.3 \pm 0.8$ mV; $k = 9.5 \pm 0.2$ mV, $n = 7$) (Fig. 4B).

These findings are similar to the previously reported profiles of γ subunits. However, they change not only the rates of deactivation, but also the half-maximal activation potential of the homomultimeric Kv2.1 channels [9–13]. Thus, Kv6.3 has a novel profile for a γ subunit, which changes only the rate of deactivation of the α subunits.

### 3.4. Biochemical association of Kv6.3 with Kv2.1

To test the notion that hKv6.3 and hKv2.1 subunits can form heteromultimeric channels, we performed an immunoprecipitation study using cells co-transfected with hKv6.3 and hKv2.1. Both subunits were expressed in COS7 cells and their association was shown by co-immunoprecipitation. To allow the immunoprecipitation of hKv6.3 protein, a 'FLAG-tag' sequence was added to the N-terminal of the hKv6.3 coding sequence. From co-transfected cells, a major band was detected by anti-FLAG or anti-hKv2.1 antibodies. These major bands with an expected molecular mass correspond to FLAG-hKv6.3 and hKv2.1 (data not shown). No signal was obtained from control transfected COS7 cells (data not shown). From co-transfected cells, hKv6.3 and hKv2.1 were co-precipitated by anti-FLAG antibodies (anti-'FLAG-tag' hKv6.3) (Fig. 5). These results clearly demonstrate the physical association of hKv6.3 and hKv2.1 subunits.

### 3.5. Summary

In this report, we describe the cloning and expression of Kv6.3, a novel member of the voltage-gated $K^+$ channel family, which was identified by searching a human genome sequencing database. Kv6.3 does not form a functional voltage-gated ion channel when expressed by itself, but when Kv6.3 and Kv2.1 were co-expressed, the heteromultimeric channels display a decreased rate of deactivation compared to that of the homomultimeric Kv2.1 channels. These profiles suggested that Kv6.3 is a γ-subunit which does not form functional channels on its own but modulates the properties of other channel subunits, for example Kv2.1, in heteromultimeric channels.

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