Identification and cloning of TWIK-originated similarity sequence (TOSS): a novel human 2-pore K^+ channel principal subunit

David J. Pountney[a,*], Iosif Gulkarov[a], Eleazar Vega-Saenz de Miera[b], Douglas Holmes[a], Michael Saganich[b], Bernardo Rudy[b], Michael Artman[a], William A. Coetzee[a]

[a] *Department of Pediatric Cardiology, New York University School of Medicine, 560 First Avenue, New York, NY 10016, USA*
[b] *Department of Physiology and Neuroscience, New York University School of Medicine, 560 First Avenue, New York 10016, USA*

Received 24 March 1999

Abstract We have identified and cloned a new member of the mammalian tandem pore domain K^+ channel subunit family, TWIK-originated similarity sequence, from a human testis cDNA library. The 939 bp open reading frame encodes a 313 amino acid polypeptide with a calculated Mr of 33.7 kDa. Despite the same predicted topology, there is a relatively low sequence homology between TWIK-originated similarity sequence and other members of the mammalian tandem pore domain K^+ channel subunit family group. TWIK-originated similarity sequence shares a low ($<30\%$) identity with the other mammalian tandem pore domain K^+ channel subunit family group members and the highest identity (34%) with TWIK-1 at the amino acid level. Similar low levels of sequence homology exist between all members of the mammalian tandem pore domain K^+ channel subunit family. Potential glycosylation and consensus PKC sites are present. Northern analysis revealed species and tissue-specific expression patterns. Expression of TWIK-originated similarity sequence is restricted to human pancreas, placenta and heart, while in the mouse, TWIK-originated similarity sequence is expressed in the liver. No functional currents were observed in *Xenopus laevis* oocytes or HEK293T cells, suggesting that TWIK-originated similarity sequence may be targeted to locations other than the plasma membrane or that TWIK-originated similarity sequence may represent a novel regulatory mammalian tandem pore domain K^+ channel subunit family subunit.

Key words: Cloning; K^+ channel; Pancreas; P-domain

1. Introduction

Potassium channels form the largest and most diverse family of ion channels in terms of both their molecular structure and function [1,2] and play a central role in many cellular functions of excitable and non-excitable cells. Homo- and heteromultimeric assembly of principal and regulatory subunits gives rise to a vast range of active channels tailored to the precise physiological function of a specific cell-type. Initially, two main groups of potassium channel principal subunits were identified in mammals, voltage-gated K^+ channel subunits possessing six transmembrane domains and a pore-forming P-domain (Kv, slo, SK, eag and KQT families) and inwardly rectifying K^+ channel principal subunits possessing two transmembrane domains flanking a conserved P-domain (Kir family). Recently, a third group of K^+-selective pore-forming principal subunits was identified in mammals. They are unique in exhibiting a novel topology of four transmembrane domains and two P-domains within a single continuous polypeptide (K2P family). These novel channel-forming subunits include TWIK (tandem of P-domains in a weak inward rectifying K^+ channel) [3], TREK (TWIK-1-related K^+ channel) [4], TASK (TWIK-1-related acid-sensitive K^+ channel) [5] and TRAAK (TWIK-related arachidonic acid-stimulated K^+ channel) [6]. Except for the P-domains, there is a low level of amino acid similarity between these subunits (30–34%). Channels formed by some members of the K2P family elicit currents that are often referred to as 'leak' or 'background' currents due to the absence of rectification properties (other than those imposed by electro-diffusional considerations) and the lack of a discernable time-dependence of whole cell currents. Database analysis indicates that this K2P family is common to the genomes of many organisms. For example, over 50 2-P channels have been identified in the nematode *Caenorhabditis elegans* [7,8], where they represent the most abundant class of K^+ channel subunits. In *C. elegans*, K2P subunits appear to have a highly defined expression pattern, restricted in certain cases to single cells such as interneurons [9].

Here, we report the cloning, tissue distribution and electrophysiological properties of hTOSS, a TWIK-originated similarity sequence from a human testis cDNA library, which is expressed predominantly in the pancreas, placenta, testis and heart.

2. Materials and methods

2.1. Cloning strategy

Using 120 bp of the TWIK-1 clone as a query sequence (corresponding to 40 amino acids spanning the P1 domain), an expressed sequence tag (accession number AA604914) was identified using the BLAST algorithm from the non-redundant EST division of GenBank [10]. A subsequent BLAST-X search of the available databases confirmed that the AA604914 nucleotide sequence was unique in that it had no identity with any other known protein. The AA604914 clone was obtained from GenomeSystems (but has been subsequently withdrawn from the I.M.A.G.E. Consortium Collection due to phage contamination at source). The full encoding sequence of 939 bp was obtained by 5′ RACE (rapid amplification of cDNA ends) PCR [11], using human testis adaptor-ligated cDNA as template (Clontech), and was sequenced on both strands.

2.2. Northern analysis

A 450 bp fragment of hTOSS (spanning the nucleotide sequence encoding the P1 domain) was labelled with [^{32}P]CTP using random primers (Boehringer). A human multiple tissue Northern blot (Clontech) was hybridized with ExpressHyb solution (Clontech) containing 1×10^6 cpm/ml. A rat probe of similar size and position was used to

*Corresponding author. Fax: (1) (212) 263 1393.
E-mail: david.pountney@med.nyu.edu

PII: S0014-5793(99)00495-0

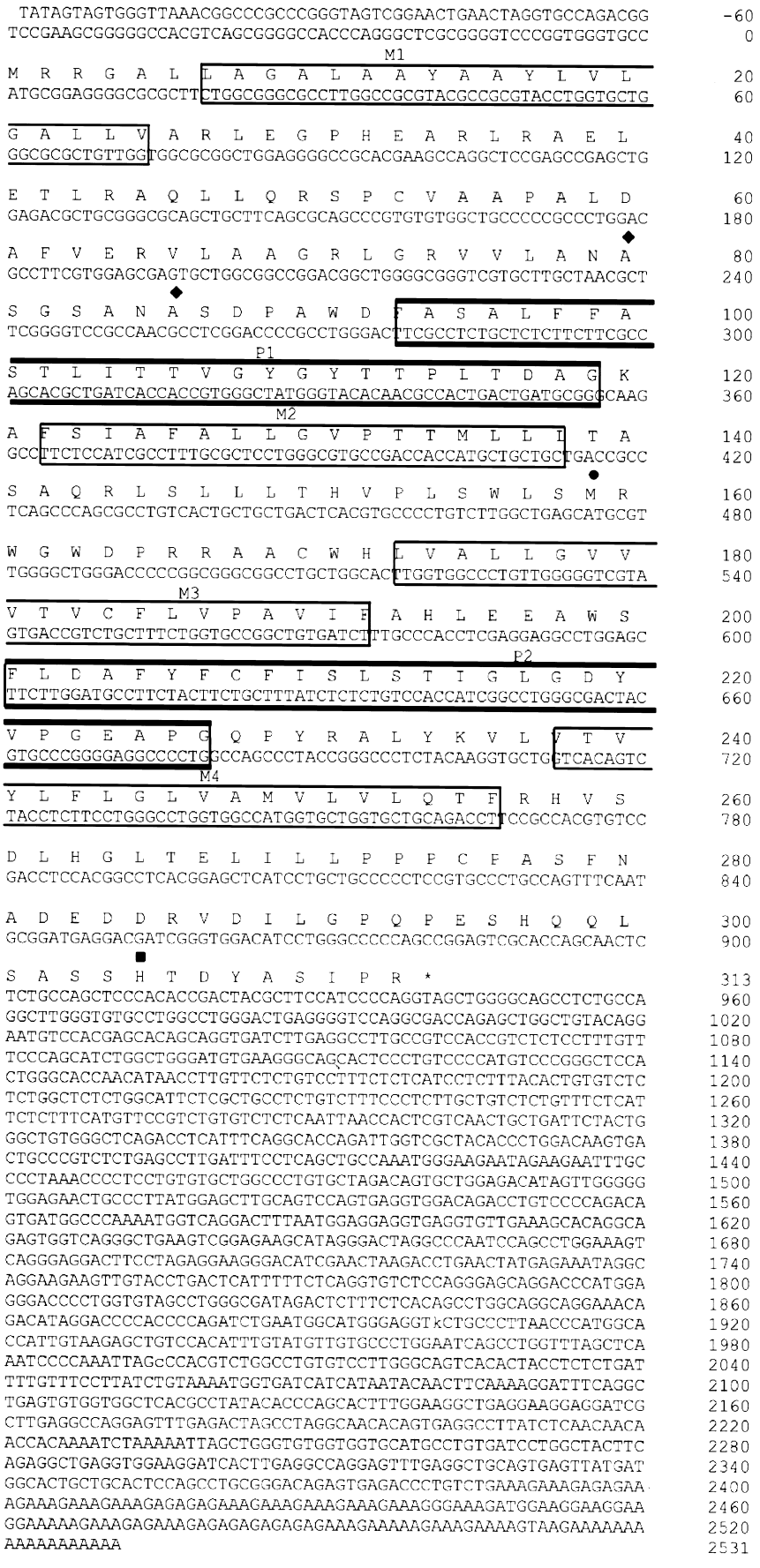
M1
P1
M2
M3
P2
M4

Fig. 1. Nucleotide and deduced amino acid sequence of hTOSS. The hTOSS cDNA sequence and the corresponding amino acid sequence translated from the 939 bp open reading frame. Putative transmembrane spanning domains (as determined by hydropathy analysis) are outlined and P-domains are indicated by gray lines above the amino acid sequence. Putative sites for *N*-glycosylation (♦), protein kinase C phosphorylation (●) and casein kinase II phosphorylation (■).
←

analyze a mouse multiple tissue Northern blot (Clontech). The blots were washed stepwise to a final stringency of 0.1×SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.3% SDS at 55°C and exposed to BioMax film (Kodak) at −80°C for 1–4 days. Blots were stripped by heating to 95°C in 0.5% SDS (w/v) for 10 min. A β-actin probe was used to assess equal gel loading.

2.3. Two electrode voltage clamp of Xenopus laevis oocytes

The encoding sequence of hTOSS was subcloned into the *Xenopus* expression vector pSP36T containing the 5′ and 3′ untranslated regions of the *Xenopus* β-globin gene and the SP6 promoter to drive transcription. hTOSS-pSP36T was linearized with *Eco*RI and cRNA was transcribed in vitro using the SP6 promoter (Stratagene). cRNA from the TWIK-1 cDNA clone was prepared in a similar manner (gift from Dr Lazdunski). Stage IV–V *Xenopus* oocytes were harvested, collagenase treated (10 mg/ml) and micro-injected with 50 nl cRNA (∽20 ng). All electrophysiology experiments were carried out at room temperature (22°C), 1–5 days following injection. Recordings were made in solutions containing (in mM) 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES pH 7.5 and either 96 Na^+-glutamate, 2 K^+-glutamate (low K^+) or 2 Na^+-glutamate, mM K^+-glutamate (high K^+). Square voltage clamp pulses were applied from a holding potential of 0 mV for 400 ms to test potentials ranging from −110 mV to +80 mV in 10 mV increments, with a 5 s interpulse duration.

2.4. Patch-clamp recordings in transfected HEK293T cells

hTOSS was subcloned into the mammalian expression vector pcDNA3 (Invitrogen) under the transcriptional control of the pCMV promoter. HEK293T cells were transiently co-transfected with hTOSS-pcDNA3 (1.65 μg) and pEGFP-C3 (0.4 μg) (Clontech) using lipofectAMINE PLUS (Gibco BRL) at 60% confluency per 35 mm plate. Whole cell recordings of GFP-positive cells were made thereafter at 24, 48 and 72 h at room temperature (22°C). A 200 ms voltage ramp protocol from +50 to −120 mV was used. Extracellular bath solution contained (in mM) 100 NaCl and 40 KCl, pH 7.4. The pipette solution contained (in mM) 140 L-aspartic acid, 10 EGTA, 5 ATP, 5.92 $MgCl_2$, 10 HEPES, pH 7.2. Acquisition of data and subsequent analysis was performed using the pClamp suite of software (Axon Instruments).

3. Results and discussion

3.1. Cloning and sequence analysis of hTOSS

An EST (AA604914) was identified in the GenBank database using the BLAST algorithm with the P-domain of TWIK-1 as query sequence [10]. Following the sequencing of this EST clone, the full encoding sequence of 939 bp was obtained by 5′ RACE PCR using human testis adaptor-ligated cDNA as template (Clontech). It was found to encode a 313 amino acid polypeptide, which we have named TOSS (Fig. 1). The nucleotide sequence of hTOSS was submitted to the GenBank and assigned the accession number AF134149. The partial sequence of a rat homologue, rTOSS, was amplified by degenerate PCR and has been submitted to the GenBank.

A strong Kozak initiation sequence was found adjacent to the start codon of hTOSS [12]. The amino acid sequence contains four putative transmembrane regions and two P-domains. The first P-domain contains a 'GYG' motif which is strongly conserved in the P-regions of most K^+ channel subunits. In contrast, the P2 domain contains a 'GLG' motif in a position corresponding to that found in TWIK-1. TASK, TREK and TRAAK contain a 'GFG' P2 motif. A hydrophobicity plot [13] shown in Fig. 2A suggests four potential transmembrane segments (M1–M4). Consistent with the lack of a signal peptide sequence, the NH_2-terminus is predicted to lie on the cytoplasmic side of the plasma membrane.

Analysis of the hTOSS amino acid sequence against the PROSITE database (http://www.expasy.ch/) reveals two putative *N*-glycosylation sites (positions 79–82 and 85–88 in the M1-P1 linker), indicating that if hTOSS is targeted to the plasma membrane, then, this large loop lies on the extracellular surface of the membrane. Phosphorylation and dephosphorylation of specific amino acid residues is often an important mechanism of ion channel regulation [14]. A consensus protein kinase C phosphorylation site is identified in hTOSS at position 158–160 in the amino acid sequence. Interestingly, TWIK-1 contains a consensus site for protein kinase C phosphorylation at a similar position [3,15]. There is also a putative casein kinase II phosphorylation site in the C-terminus of hTOSS at position 304–307.

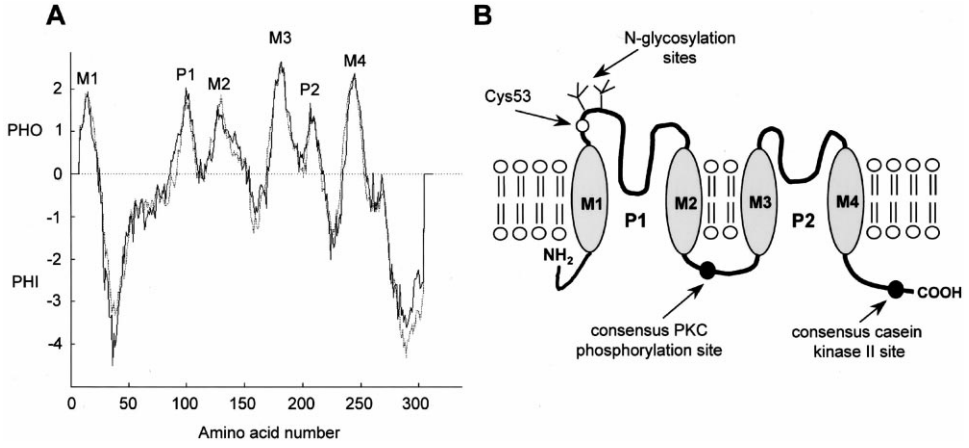

Fig. 2. Hydropathy analysis and deduced topology for hTOSS. (A) Hydrophobicity was determined according to the method of Kyte and Doolittle [13] using a window size of 17 amino acids (www.isrec.isb-sib.ch/). (B) Predicted topology. Consensus sites for N-linked glycosylation, phosphorylation by protein kinase C and casein kinase II are indicated.

3.2. Absence of TOSS currents in heterologous expression systems

In oocytes injected with hTOSS cRNA, the membrane currents recorded were not significantly different to background currents in water-injected eggs (Fig. 3A and B). Changing the voltage protocol by holding at −120 mV and depolarizing in 20 mV steps from 120 to −160 mV (400 ms duration) also failed to elicit currents larger than those recorded from H_2O-injected oocytes. As a positive control, we were routinely able to measure currents from oocytes injected with Kir 2.1 cRNA. The ability of hTOSS cRNA to be transcribed in vitro was verified using the rabbit reticulocyte lysate system (results not shown).

Initial attempts to heterologously express hTOSS cDNA under the control of the pCMV promoter in HEK293T cells similarly failed to demonstrate significant currents above background, even 72 h post-transfection (50–200 pA currents were observed in both transfected and non-transfected cells). These observations raise several interesting possibilities. hTOSS subunits may be targeted to membranes other than the plasma membrane. Alternatively, hTOSS may possess a regulatory function, modulating the properties of other principal channel-forming subunits with tissue-specific implications.

3.3. Distribution of hTOSS mRNA

TOSS expression is localized to specific tissues. In the mouse (Fig. 4A), a band of 2.4 kb is detected only in the liver. In contrast, in human (Fig. 4B), there is no signal in liver but there are several bands of 1.35, 2.4 and 6 kb in the pancreas, a 2.4 kb band in the placenta and a band of 6 kb is present in the heart. These bands may represent alternatively spliced transcripts. In contrast, TWIK-1 is expressed in a wide range of tissues including the brain, placenta and pancreas of both human and mouse and in the human heart [3,16].

Both voltage-gated and inwardly rectifying K^+ channels are thought to be a multimeric assembly of four principal subunits [17,18]. Tetramerization of a single P-domain containing subunits is believed to occur via interaction in the N- and/or C-terminus [18]. Interestingly, the amino acid sequence of hTOSS also contains a cysteine residue in a similar position in the M1-P1 loop (Fig. 2B). While the mechanism underlying the assembly of K2P principal subunits is unknown, there is evidence that TWIK-1 can co-assemble to form dimers via an

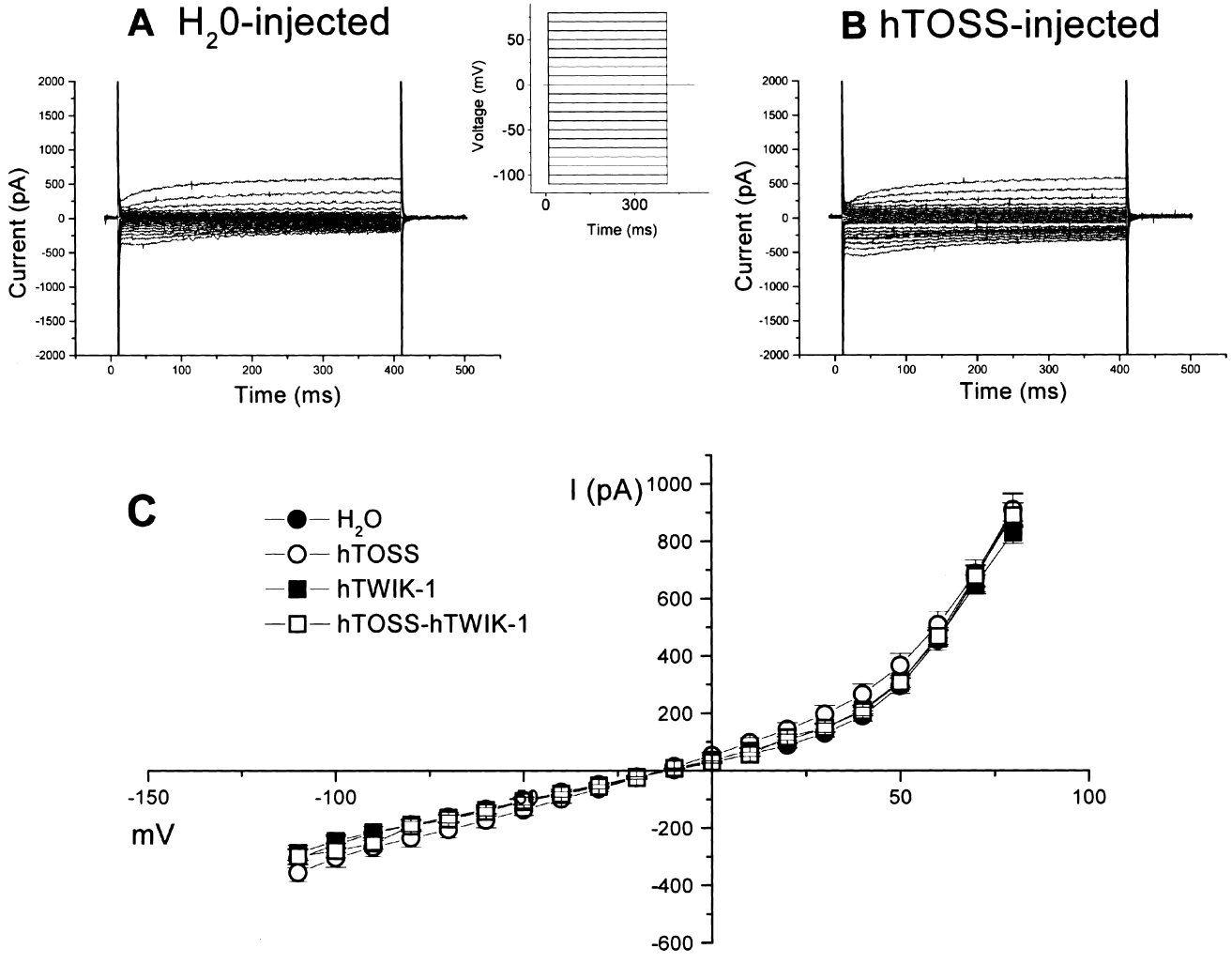

Fig. 3. Expression of hTOSS in *X. laevis* oocytes. Steady state current-voltage (I–V) relationships of currents recorded in 96 mmol/l $[K^+]_o$ from oocytes injected with either H_2O (●), hTOSS cRNA (○), hTWIK-1 cRNA (■) or equimolar concentrations of hTOSS and hTWIK-1 cRNA (□). Further experimental details are given in the text.

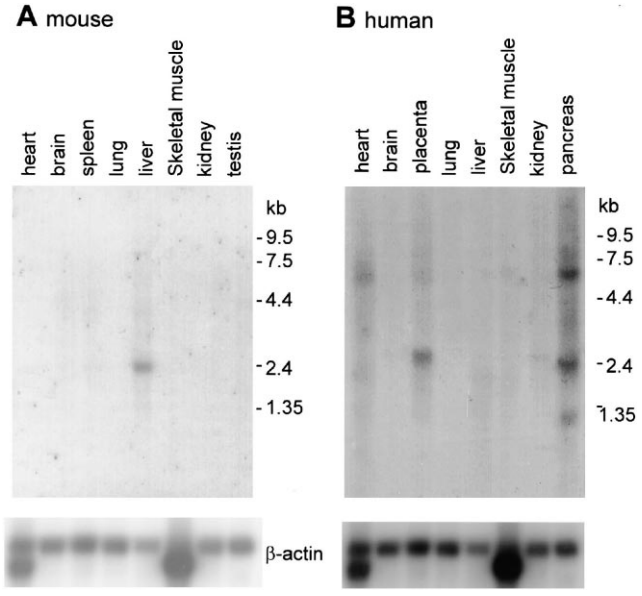

Fig. 4. Distribution of TOSS mRNA in human and mouse tissues. (A) A mouse multiple tissue Northern blot (MTN, Clontech) was probed at high stringency with a rat TOSS cDNA probe. (B) A human multiple tissue Northern blot (MTN, Clontech) was probed at high stringency with a human TOSS cDNA probe. The cDNA probes spanned the nucleotide sequence encoding the P1 region. Each lane contained 2 μg of poly(A^+) RNA. The exposure time was 4 days. The blots were subsequently stripped and analyzed with a β-actin probe as a control (lower panels).

inter-chain disulfide bridge involving a cysteine residue in the M1-P1 linker loop [19]. This particular cysteine residue is conserved in TREK-1 [4] but not in TASK [5], suggesting that its presence is not an absolute requirement for assembly of K2P subunits.

Of the K2P subunits cloned to date, hTOSS shares the highest level of similarity with hTWIK-1 (Fig. 5), suggesting that TOSS and TWIK may be co-assembly partners. This suggestion is strengthened when considering the overlap of their tissue distribution (heart, pancreas and placenta). We consequently performed experiments to test this possibility. We were unable to detect currents above background in oocytes expressing hTOSS. Despite the initial report that TWIK may express weakly inwardly rectifying currents [3], we were unable to demonstrate a significant current in oocytes injected with TWIK-1 cRNA. This finding is consistent with the findings of others [20] who have also been unable to demonstrate TWIK-1 currents in *Xenopus* oocytes. Co-injection of equimolar concentrations of TWIK-1 and TOSS cRNA also failed to generate currents when two electrode voltage clamping was performed (Fig. 3C), indicating that co-expression of TWIK-1 and hTOSS does not result in functional channels.

TOSS represents the fifth cloned member of the emerging mammalian family of 2-pore K^+ channel principal subunits. It seems likely that in mammals, further members of this new and expanding K2P subfamily remain to be identified. Considering the specific tissue distribution of hTOSS and other K2P family members, potentially novel K2P subunits with which TOSS may interact may be significantly under-represented in the current genomic and cDNA databases and libraries. While it is premature to hypothesize the function of K2P K^+ channels in normal or pathophysiological conditions, their limited distribution suggests that they may serve to specifically modulate the K^+ conductance in the cells in which they are expressed. By identifying and characterizing novel K2P subunits, the potential therapeutic targets for disorders of K^+ channel function is increased. Further studies are currently underway to evaluate specific cell-type expression patterns and to identify other principal subunits with which TOSS may interact to form or modulate active channels.

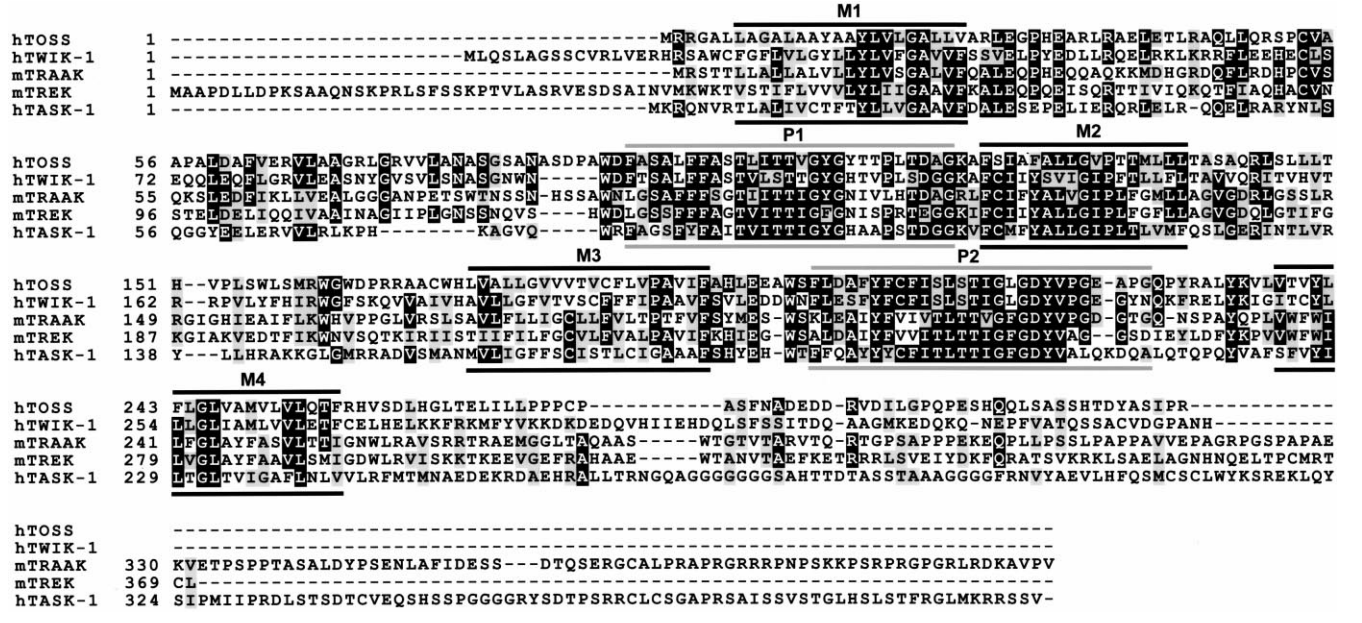

Fig. 5. Alignment of mammalian two P-domain principal subunit amino acid sequences. Amino acid sequences were aligned using Clustal-W, using the 'blosum' weight matrix, a gap opening penalty of 20 and a gap extension penalty of 0.1. Conserved and similar residues are shaded by Boxshade software (www.isrec.isb-sib.ch, version 2.5)

Acknowledgements: We wish to thank Drs Lisa Porter and Peter Haddock for the technical assistance and valuable discussion. The nucleotide sequence data reported in this article have been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AF134149.

References

[1] Coetzee, W.A., Amarillo, Y., Chui, J., Chow, A., Lau, D., McCormack, T., Moreno, H., Nadal, M., Ozaita, A., Pountney, D.J., Saganich, M., Vega-Saenz de Miera, E. and Rudy, B. (1999) New York Academy of Sciences (in press).
[2] Rudy, B. (1988) Neuroscience 25, 729–749.
[3] Lesage, F., Guillemare, E., Fink, M., Duprat, F., Lazdunski, M., Romey, G. and Barhanin, J. (1996) EMBO J. 15, 1004–1011.
[4] Fink, M., Duprat, F., Lesage, F., Reyes, R., Romey, G., Heurteaux, C. and Lazdunski, M. (1996) EMBO J. 15, 6854–6862.
[5] Duprat, F., Lesage, F., Fink, M., Reyes, R., Heurteaux, C. and Lazdunski, M. (1997) EMBO J. 16, 5464–5471.
[6] Fink, M., Lesage, F., Duprat, F., Heurteaux, C., Reyes, R., Fosset, M. and Lazdunski, M. (1998) EMBO J. 17, 3297–3308.
[7] Wei, A., Jegla, T. and Salkoff, L. (1996) Neuropharmacology 35, 805.
[8] Ketchum, K.A., Joiner, W.J., Sellers, A.J., Kaczmarek, L.K. and Goldstein, S.A. (1995) Nature 376, 690–695.
[9] Salkoff, L., Kunkel, M.T., Butler, A., Wang, Z.-W., Nonet, M.L. and Wei, A. (1999) Biophys. J. 76, A414.
[10] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[11] Zhang, Y. and Frohman, M.A. (1997) Methods Mol. Biol. 69, 61–87.
[12] Kozak, M. (1996) Mamm. Genome 7, 563–574.
[13] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[14] Levitan, I.B. (1994) Ann. Rev. Physiol. 56, 193–212.
[15] Lesage, F., Lauritzen, I., Duprat, F., Reyes, R., Fink, M., Heurteaux, C. and Lazdunski, M. (1997) FEBS Lett. 402, 28–32.
[16] Cluzeaud, F., Reyes, R., Escoubet, B., Fay, M., Lazdunski, M., Bonvalet, J.P., Lesage, F. and Farman, N. (1998) Am. J. Physiol. 275, C1602–C1609.
[17] Doyle, D.A., Cabral, J.M., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T. and MacKinnon, R. (1988) Science 280, 69–77.
[18] Yang, J., Jan, Y.N. and Jan, L.Y. (1995) Neuron 15, 1441–1471.
[19] Lesage, F., Reyes, R., Fink, M., Duprat, F., Guillemare, E. and Lazdunski, M. (1996) EMBO J. 5, 6400–6407.
[20] Goldstein, S.A., Wang, K.-W., Ilan, N. and Pausch, M. (1998) J. Mol. Med. 76, 13–20.