Tpr1, a *Schizosaccharomyces pombe* protein involved in potassium transport

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Abstract **The *Schizosaccharomyces pombe Tpr1* was isolated as suppressor of the *Saccharomyces cerevisiae Δ trk1,2* potassium uptake deficient phenotype. *Tpr1*, for tetratrico peptide repeat, encodes a 1039 amino acid residues protein with several reiterated TPR units displaying significant homology to p150^{TSP}, a recently identified phosphoprotein of mouse, to *S. cerevisiae* CTR9 and to related sequences of human, *Caenorhabditis elegans*, *Methanoccocus jannaschii* and *Arabidopsis thaliana*. Expression of *Tpr1* restored growth on 0.2 mM K^+ media, induced K^+ transport with a K_T of 4.6 mM and resumed inward currents of −90 pÅ at −250 mV (pH 7.2) conducting K^+ and other alkali-metal ions. The tetratrico peptide repeat is a degenerate motif of 34 amino acids that is repeated several times within TPR-containing proteins and has been suggested to mediate protein-protein interactions. The sequence and putative binding properties of Tpr1 suggest the protein unlikely as transporter but involved in the enhancement of K^+ uptake via conventional carriers.**

Key words: Potassium transport; TPR motif; Whole-cell patch-clamp analysis; *Schizosaccharomyces pombe*

1. Introduction

Transport of potassium ions is an essential process for most cells. In fungi and plants, specific transporters take up sufficient levels of potassium from the environment to accommodate intracellular ionic requirements. In *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, potassium uptake is accomplished by specific TRK transporters [14,17,27]. *S. cerevisiae* strains containing mutations in both transporters are severely impaired in K^+ uptake and require media supplemented with high concentrations of KCl for growth. Suppression of the growth defect of these strains has revealed a large number of heterogeneous genes, encoding such diverse proteins as plant and vertebrate inward-rectifying K^+ channels [2,29,31,21,34], a plant K^+ transporter [30], a two pore domain K^+ selective ion channel of *Drosophila melanogaster* [11], ATPases [23,24], and among *S. cerevisiae* suppressors, glucose transporters [15] and amino acid permeases [35]. Complementation of potassium uptake mutants of *S. cerevisiae* with *S. pombe* cDNAs has previously identified the *S. pombe* potassium transport protein TKHp [17]. Here we describe the isolation and functional analysis of the *Tpr1* gene from the fission yeast *S. pombe* whose expression complements the *trk1 trk2* mutant phenotype of *S. cerevisiae* strain CY162 [14], which is defective in potassium uptake. Tpr1p (for tetratrico peptide repeat) contains within its amino-terminal portion a tandem array of 16 TPR units, displaying high overall similarity to related proteins of *S. cerevisiae* [8], mouse [18], *Caenorhabditis elegans* [33], *Arabidopsis thaliana* [13], *Methanoccocus jannaschii* [7] and human [22]. The sequence and functional properties of Tpr1p suggest that it may be involved in protein-protein interactions thereby triggering potassium uptake via conventional transporters.

2. Materials and methods

2.1. Yeast strains, media, plasmids and molecular methods

All yeast strains were grown at 30°C. *S. cerevisiae* strain CY162 (*Matα ura3–52 his4–15 his3Δ200 trk1Δ trk2Δ::HIS3*) was grown in complete synthetic medium consisting of 2% D-glucose, 100 mM KCl and supplemented with uracil (Sigma, USA). Selective media contained 0.2 to 7 mM KCl without uracil appropriate for selection and maintenance of plasmids in the transformed strains. The *S. pombe* cDNA library was obtained from Dr. F. Lacroute (CNRS, Gif sur Yvette, France) and constructed as described [19] under control of the constitutive phosphoglycerate kinase promotor. All transformations, including the cDNA library, were performed with freshly prepared yeast cells using the electroporation technique [3]. Plasmid recovery from transformed strains was performed as described [12]. *Escherichia coli* strain XL-1BLUE was used as host for propagation and construction of plasmids. Plasmid pUCT92 contained the *SPTpr1* gene on a *Not*I fragment which was ligated with *Sma*I digested pUC18 (Roche Molecular Biochemicals) following fill-in of the protruding ends with the Klenow fragment of DNA polymerase. Sequences were determined using the dye-chain-termination method (Cy5-Autoread Kit, Pharmacia) on both strands. The sequence of *Tpr1* has been assigned the accession number AF047464. Plasmid BST92 contained *SPTpr1* as a *Not*I fragment under the control of the T7 promotor in vector pBSK (Stratagene). In vitro transcription/translation was performed with the TNT Coupled Reticulocyte Lysate Systems used together with the tRNA^{nscend} Non-Radioactive Translation Detection System (Promega/Serva) according to manufacturers recommendations. For Southern and Northern analysis the 0.96 kb *Hin*dII fragment of the coding region was radiolabelled by random priming (Megaprime, Amersham) and used as probe. Chromosomal DNA (10 μg) from *S. pombe* was digested, separated on 0.8% agarose gels, and transferred to Nylon membranes by standard procedures. RNA isolated as described [20] and Northern blot analysis was performed according to [28] with 20 μg total RNA. Hybridisation was performed at 42°C in hybridisation buffer (5×SSC, 5×Denhardt's solution, 50% formamide and 1% SDS) for 18 to 24 h. For integrity and abundance of the isolated RNA a 389 bp fragment of the *Pma1* gene of *S. pombe*, encoding the plasma membrane ATPase, served as control for reprobing of the blots. The *Pma1* probe was derived from polymerase chain reactions involving *S. pombe* genomic DNA and specific primers (2557–2585: 5′ CCTTACCAAGAACAAGTTGTCTCTTGGTG 3′ and 2917–2946: 5′ GAACGATAACCACGAGAAGCCAAATCACCG 3′). The sizes of the messages were determined relative to the mobility of the 9.5–0.24 kb ladder from Gibco BRL.

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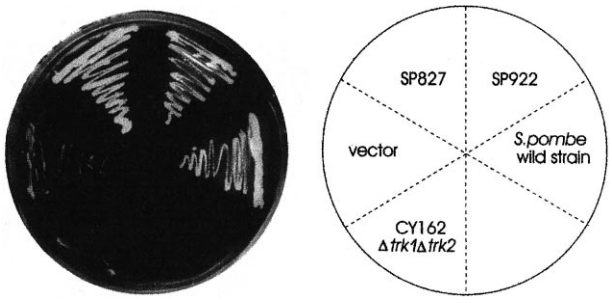

Fig. 1. Functional complementation of K^+ transport defective *S. cerevisiae* cells by *S. pombe* Tpr1p. Wild-type strain: *S. pombe* 972 h^-; CY162: *S. cerevisiae trk1 trk2* mutant strain; Vector: CY162 cells containing the pFL61 (PGK promoter) plasmid; SP922: expressing *S. pombe Tpr1*; SP827: expressing the *S. pombe* K^+ transporter TKHp. Minimal selective medium with 7 mM KCl; growth for 2 days at 30°C.

2.2. Uptake experiments

In vivo K^+ uptake analysis was carried out as described in [17] with a potassium-specific electrode (Orion 931900) with constant agitation of the cell suspension for 10 to 15 min at 25°C.

2.3. Yeast electrophysiology

Growth of the yeast cells, preparation of spheroplasts and the patch-clamp recording in the whole-cell configuration were performed as described originally [4,5]. The preparation procedure yielded protoplasts of 5–10 μm in diameter for *S. cerevisiae* wild-type cells, and of 3–5 μm in diameter for CY162 cells and the *Tpr1* expressing SP922 strain. For patch-clamp recording the whole-cell configuration was achieved either spontaneously during the seal formation or subsequently through suction or voltage pulses (600 mV, 25 ms). Giga-ohm (1 and 10 GΩ) seals were obtained using glass micropipettes (Kimble, Kimax-51) of resistances between 3–5 MΩ. The bath solution contained either 150 mM, 175 mM, or 200 mM KCl along with 5 mM $MgCl_2$, 10 mM $CaCl_2$ in 100 mM Tris/MES (pH 7.5 or pH 5.5). The pipette solution contained 175 mM KCl, 5 mM $MgCl_2$, 4 mM ATP, 0.14 mM $CaCl_2$, 1 mM EGTA (pH 7.0 adjusted with KOH). The transmembrane current was amplified using a BLM-10 preamplifier and BLM-120 amplifier (both Biologic, Echirolles, France), filtered at 1 kHz through an 8-pole Bessel filter (Frequency Devices, model 902, Compu-Mess-Elektronik GmbH, Garching), and fed through a TL-1 Interface (Axon instruments Inc., Foster City, CA) to an IBM-AT computer. The experiment was controlled through a p_{CLAMP} programme (ver. 5.0, Axon Instruments Inc.). Measured currents were monitored in response to 2700 ms voltage steps progressing from +100 to −200 mV by 20 mV increments including a 300 ms holding interval at −40 mV, analysed by the computer and plotted using Origin 5.0 software (Microcal, Northampton, MA). The current/voltage curves were corrected for leak current by using Excel 7.0 software (Microsoft Corp.). Leak currents were assessed from the linear part of the current/voltage curves between +20 and −100 mV as carried out previously [17]. It varied between 100–300 nÅ/mm^2 per 100 mV depending on ion composition of the bath solution and quality of the giga-ohm seal. The flow of cations across the plasma membrane into the protoplast was designated inward current and considered negative.

3. Results

3.1. Isolation and sequence analysis of the S. pombe Tpr1

For isolation of *S. pombe* genes involved in potassium transport a *S. pombe* cDNA library [19] was screened for suppression of the *S. cerevisiae* K^+ uptake deficient mutant CY162 (*MATα ura3–52 his4–15 his3Δ200 trk1Δ trk2Δ:HIS3*). This double mutant, lacking both functional *TRK1* and *TRK2* alleles due to deletion and disruption of the two genes, respectively [14], is deficient for K^+ uptake, and thus unable to grow in a low potassium medium. From approximately 3×10^4 primary transformants 1×10^2 yeast colonies were selected by growth on 7 mM potassium medium. Further reduction

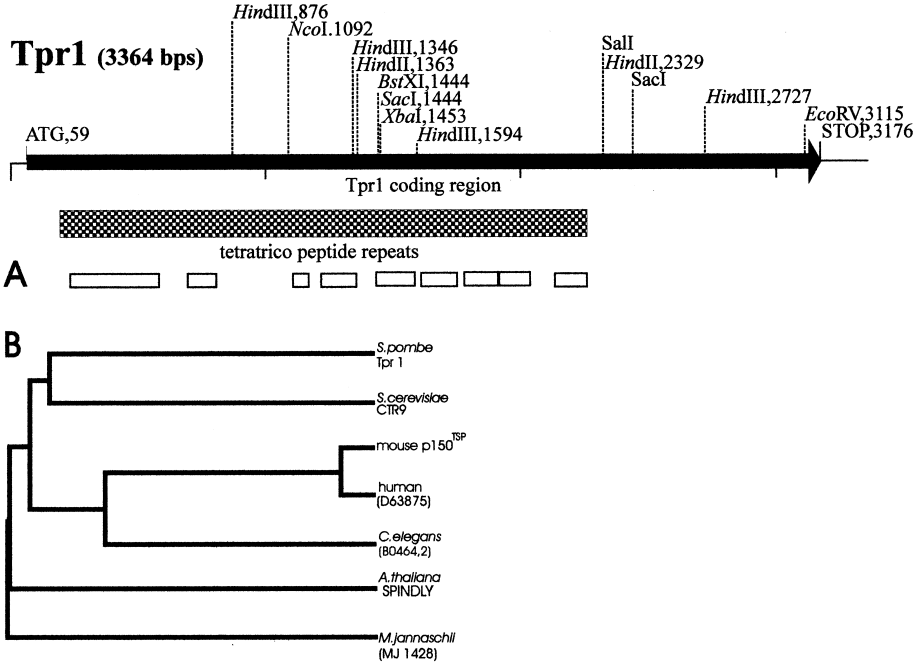

Fig. 2. Panel A: Restriction map of *S. pombe Tpr1* (AF047464) with coding region as filled arrow. The nucleotide sequence precedes an in-frame stop-codon six nucleotides upstream of the putative ATG start-codon. Shaded box: 16 predicted repeats of the tetratrico peptide (TPR) motif between amino acid residues 50 and 736 within Tpr1. Open boxes: Relation of predicted α helices to the location of the tetratrico repeats. Panel B: Dendrogram taken from the amino acid alignment of *S. pombe* Tpr1 and homologues with TPR consensus motifs. Conceptual translations of the *C. elegans* Q03560, the human D63875, and the *M. jannaschii* U67584 open reading frames were aligned with *S. cerevisiae* CTR9, mouse $p150^{TSP}$, *A. thaliana* SPINDLY and *S. pombe* Tpr1.

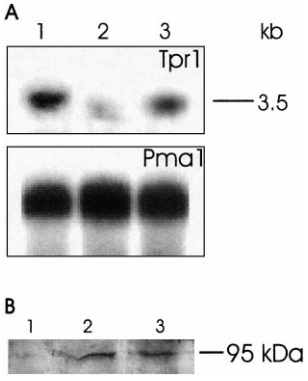

Fig. 3. A: *Tpr1* is a single RNA species of 3.5 kb. 20 μg of total RNA from: *S. pombe* wild-type cells (lane 1), *S. cerevisiae trk1 trk2* mutant CY162 (lane 2) and SP922 (lane 3), was electrophoretically separated, blotted onto Nylon membranes and probed with high stringency using the 0.96 kb *Hin*dII fragment of the *Tpr1* coding region. The weak corresponding signal in CY162 may be due to the CTR9/CDP1 transcript. For integrity and abundance of the isolated RNA a 389 bp fragment of the *Pma1* gene of *S. pombe*, encoding the plasma membrane ATPase was used in a control blot. B: In vitro transcription/translation. Lane 1: negative control; lane 2: product of *S. pombe Tpr1* cDNA; lane 3: control product of *SpTRKH* cDNA (833 amino acids). The translated proteins were separated on a 10% SDS-PAGE gel, blotted onto nitrocellulose membranes and subsequently immunodetected with the Streptavidin-AP/Western Blue Substrate. The proteins migrated at an approximately size of 95 kDa.

of external potassium concentration to 0.2 mM KCl identified, among others, two suppressors with growth rates comparable to the wild-type on low K^+ medium. Plasmids were recovered from those yeast clones and subsequently reintroduced in the *trk1 trk2* mutant strain. The two clones contained overlapping cDNAs, according to restriction maps and nucleotide sequences, indicating that they arose independently from identical mRNA. Fig. 1 illustrates the plasmid-dependent suppression of the *trk1 trk2* conferred phenotype of strain SP922 expressing *S. pombe Tpr1* on low K^+ media which is comparable to the expression of the previously described *S. pombe* TKHp [17]. Stability tests with SP922 cells grown under non-restrictive conditions (100 mM KCl-YPD) yielded colonies unable to grow on low K^+ media again. Subcloning identified a 3364 bp *Not*I fragment with the nucleotide sequence of *S. pombe Tpr1* (AF047464) capable of encoding a polypeptide of 1039 amino acids. Between amino acid residues 50 and 736 the Tpr1 protein sequence contains 16 repeats of the 34 amino acid tetratrico peptide (TPR) motif (Fig. 2A) and multiple potential protein kinase phosphorylation sites mostly indicated within the serine, glutamate and aspartate carboxy-terminal residues distal to residue 736. Secondary structure algorithms identified no membrane associated helices with the computational translation product though 57% of the polypeptide chain was predicted as helical conformation. Analysis of nucleotide and protein sequence databases using the TBLASTN algorithm [1] revealed 44% amino acid sequence identity between *S. pombe* Tpr1 and *S. cerevisiae* CTR9 [8]. In addition, Tpr1p was found to share 30% amino acid sequence identity with a hypothetical, 1173-codon open reading frame in the human genome (D63875) [22], to the recently identified $p150^{TSP}$ of *M. musculus* [18] to SPINDLY of *A. thaliana* [13] and, to a lesser extent (26% amino acid sequence identity) to a putative 1245 amino acid protein encoded at the B0464.2 locus on chromosome III of *C. elegans* [33]. Taken from amino acid alignments the similarity between these gene products includes several of the TPR units but extends beyond it, thus supporting the hypothesis of a homologous, evolutionary conserved gene family (Fig. 2B). The homologous *M. jannaschii* sequence (U67584) [7] is with 18% overall amino acid sequence identity less related. Southern blot analysis of *S. pombe* genomic DNA probed with the 966 bp *Hin*dII fragment of *Tpr1* under high and low stringency conditions detected a single signal in three different digests indicating no gene duplication but rather a single gene (data not shown). Within the *S. pombe* genome sequencing project an identical open reading frame (AL009227) was identified on chromosome I. Northern blot analysis of *S. pombe* wild-type cells detected a single RNA species of about 3.5 kb (Fig. 3A). The transcript of the *SPTpr1* cDNA in SP922 cells appeared to be somewhat smaller than the wild-type *S. pombe* transcript suggesting that 5′ or 3′ untranslated regions are incompletely represented in the

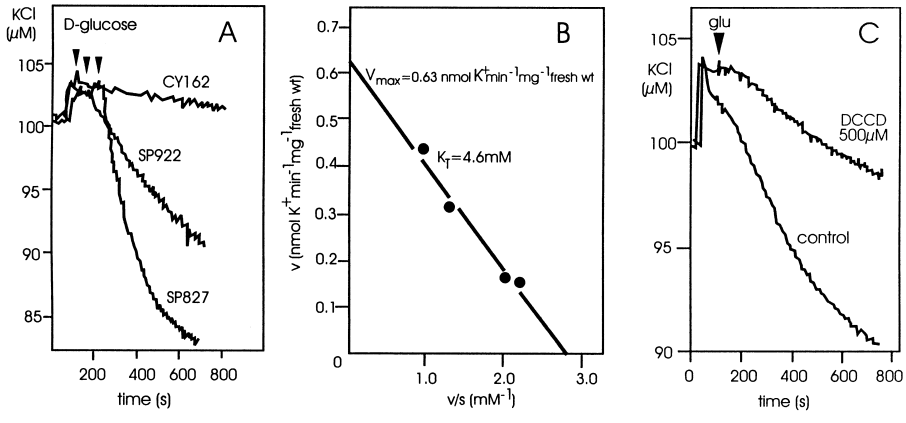

Fig. 4. Potassium consumption assays comparing *S. cerevisiae* Δ *trk1,2* strain CY162, strain SP922 expressing *S. pombe Tpr1* and strain SP827 expressing the *S. pombe* K^+ transporter TKHp. The arrows indicate the addition of glucose to start the assay. Panel A: Representative recording of K^+ uptake at an external concentration of 100μM external KCl. Panel B: Woolf-Hofstee plot of K^+ uptake in SP922 cells. A K_T of 4.6 mM and a V_{max} of 0.63 nmol K^+ min^{-1} mg^{-1} fresh wt was calculated. Panel C: Representative recordings of K^+ uptake following addition of DCCD (500 μM) to SP922 cells.

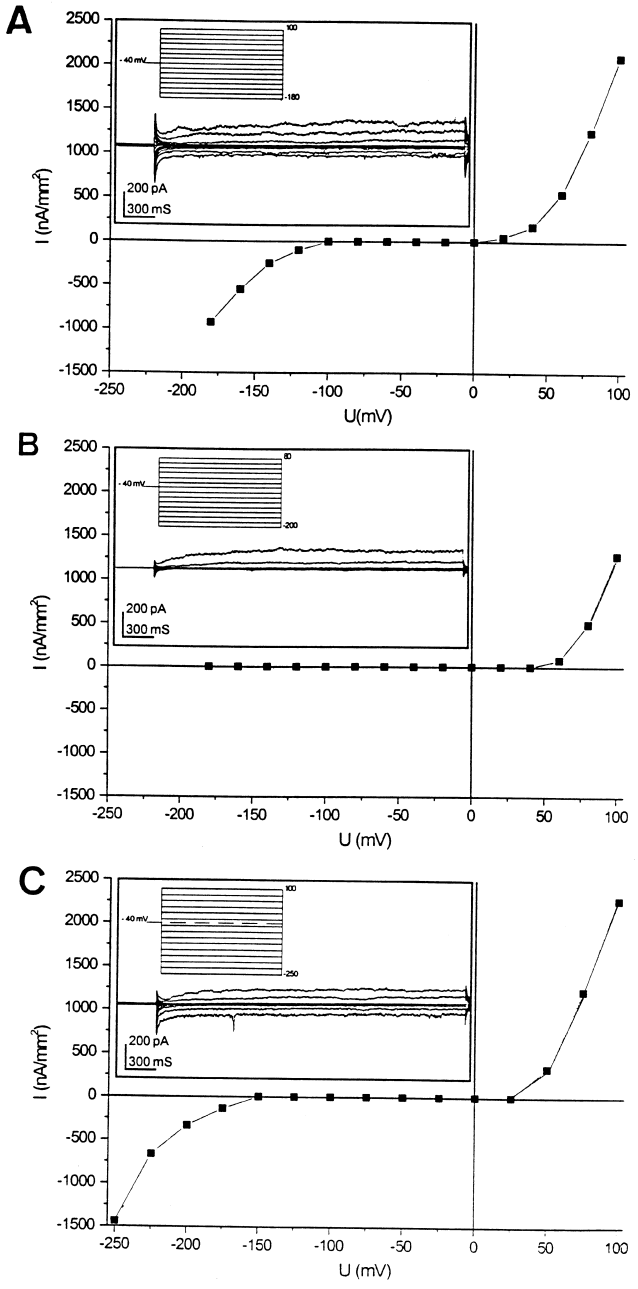

Fig. 5. Current/voltage relation of steady-state currents obtained from whole-cell patch-clamp recordings in symmetric K^+ concentrations. After seal formation of 1–10 GΩ and obtaining the whole-cell configuration the signals were filtered at 1 kHz and processed by subtracting the leak current. Panel A: *S. cerevisiae* wild-type, panel B: *S. cerevisiae Δ trk1,2* strain CY162. Voltage-clamp protocols and current traces are given in the insets. Generally, after seal formation of 1–10 GΩ and obtaining the whole-cell mode, currents were elicited by 2700 ms depolarising pulses from −180 mV to +100 mV for *S. cerevisiae* wild-type and *S. cerevisiae* Δ *trk1,2* strain CY162 from a holding potential of −40 mV (300 ms). Panel C: Current/voltage relations from steady-state currents of SP922 protoplasts. Currents were evoked by potential steps from −250 mV to +100 mV from holding potential of −40 mV. Recordings of the control *S. cerevisiae trk1 trk2* mutant strain CY162 transformed with the vector pFL61 were identical to CY162 (data not shown).

cDNA. The transcript of the *S. pombe* ATPase (*Pma1* gene) served as control for integrity and abundance of mRNA in this experiment. The predicted molecular size of the protein (ca. 119 kDa) is not consistent with the product observed upon translation of *Tpr1* cRNA (Fig. 3B); the discrepancy may arise from the anomalous electrophoretic mobility caused by the highly acidic carboxy-terminal region.

3.2. Functional analysis of the S. pombe Tpr1p

Characterisation of the *S. pombe* Tpr1p was performed in strain SP922 by kinetic analysis of potassium transport involving a K^+-specific electrode. Estimation of initial uptake rates was carried out with exponentially grown cells shifted to different external K^+ concentrations for calculation of the corresponding K_T and V_{max} values (Fig. 4A). The Woolf-Hofstee plot shown in Fig. 4B revealed a K_T of 4.6 mM for Tpr1p. Strain SP827, expressing the *S. pombe* potassium transporter *SPTRKH* served as positive control for high affinity K^+ uptake. To assess whether Tpr1p mediated K^+ uptake was dependent on the H^+-ATPase activity potassium uptake was measured in the presence of DCCD, an inhibitor of *S. pombe* H^+-ATPase [9]. Inhibition of the H^+-ATPase by DCCD at an external pH of 5.5 resulted in a significant decrease of K^+ uptake (Fig. 4C). Whole-cell patch-clamp recordings revealed in *S. cerevisiae* wild-type cells an outward current which corresponds to the endogenous K^+ channel described earlier [5,26], and an inward current activating at −100 mV (Fig. 5A) [4,5]. The outward current was taken as qualitative control in all experiments. In comparison to the *trk1 tkr2* mutant (Fig. 5B), which displayed no inward current (downward), expression of *Tpr1* regained large inward currents (Fig. 5C). Both, voltage dependency and current ampli-

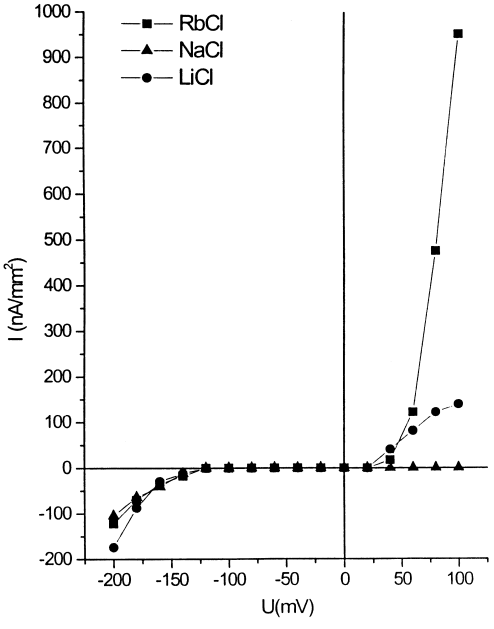

Fig. 6. Current-voltage relationships of SP922 protoplasts in the presence of different extracellular monovalent cations. Concentrations of cations in the bath solution were 150 mM. Other experimental conditions as in Fig. 5. CsCl was conducted as KCl (data not shown). The given data represent mean values of at least three measurements with S.E.M. of less than 5%.

tude of plotted inward currents of *S. cerevisiae* wild-type protoplasts are considerably different compared to those in SP922 protoplasts. The observed inward currents in SP922 spheroplasts were unusual large in that at clamped voltages of −200 mV a current of −230 nÅ/mm^2 ($n=9$) was detected, which significantly increased to −1450 nÅ/mm^2 ($n=8$) at prolonged hyperpolarisation with −250 mV. Inward currents were studied in bath solutions where KCl was replaced by isotonic substitution of NaCl, RbCl, LiCl and CsCl (Fig. 6). The inward currents carried by Na^+, Rb^+, Li^+ and Cs^+ are about 50% of those carried by K^+, −180 nÅ/mm^2 versus −500 nÅ/mm^2 at −200 mV, indicating a preference of the translocator for K^+ and almost no selectivity among other alkali-metal ions.

4. Discussion

4.1. Tpr1 function

The *S. pombe Tpr1* is an unusual suppressor of the *S. cerevisiae Δ trk1,2* potassium uptake deficient phenotype because it does not encode an integral membrane protein. In vivo potassium transport assays detected specific K^+ uptake down to concentrations of 40 μM external K^+ (data not shown) although the kinetic transport constant (K_T of 4.6 mM) indicates relative low affinity compared to *S. cerevisiae* TRK1 (K_T 0.02 mM) [27] or TRK2 (2 mM) [25] or *S. pombe* TKHp (K_T 0.8 mM) [17]. K^+ uptake is almost abolished by inhibition of the plasma membrane H^+-ATPase as would be assumed for a H^+-coupled transporter. Inward currents in SP922 spheroplasts activate at considerably more negative potentials (−150 mV) than reported for the *S. pombe* potassium transporter THKp (−100 mV) [17] or *S. cerevisiae* wild-type cells comprising TRK1 and TRK2 activity [4,5] and gain similar large conductances as the recently described NSC1 [6] raising the question of the possible mechanism by which *S. pombe Tpr1* function can account for the observed effects. Tpr1p comprises a tandem array of 16 putative TPR units. Comparison of TPRs from a variety of proteins identified 34 amino acids that form the reiterated motif, with a repeated basic structure of eight consensus residues conserved in terms of size, hydrophobicity and spacing [32]. Secondary prediction proposed a model where a single repeat is divided into two overlapping subdomains, domains A and B, forming a pair of amphipatic α helices [16]. Comparison of individual predicted TPR motifs of the *S. pombe* Tpr1p with a secondary structure prediction revealed an almost perfect overlapping pattern of helical conformations (Fig. 1, left panel) among the TPR repeats. The TPR units of Tpr1p, by forming amphipatic helices could undergo dimerisation with other proteins which contain paired helices. Potassium accumulation of *S. pombe Tpr1* expression arises likely from the high number and conservation degree of the TPR motifs mediating interspecies protein-protein interactions and thus may alter transporters of other specificity into K^+ translocators.

4.2. Tpr1p relation

Tpr1p is significantly related to six other gene products (Fig. 2B) where the high homology between Tpr1p and CTR9 of the two yeast species, but also to the orthologous proteins from mouse, human, nematode, plants and archaebacteria presumably indicate a new multiple TPR motif-containing family. Despite of the high overall homology within these proteins TPR motifs by themselves are not determining a specific biological function nor are restricted to one type of protein since many proteins identified so far to contain several blocks of the reiterated repeat carry out specific but distinct biological functions [16]. The hypothesis of a basic feature of multiple protein complex formation which could reflect the functional role of this family is supported by CTR9/CDP1 involvement in G1 cyclin expression [10] and SPINDLY involvement in signal transduction [13].

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