J. Membrane Biol. 152, 169–181 (1996)

The Journal of
Membrane
Biology

The *SpTRK* Gene Encodes a Potassium-specific Transport Protein TKH_p in *Schizosaccharomyces pombe*

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Received: 18 December 1995/Revised: 2 April 1996

Abstract. Complementary DNAs involved in potassium transport in *Schizosaccharomyces pombe* were selected by complementation of defective K^+ uptake in a *trk1 trk2* mutant of *Saccharomyces cerevisiae.* Here we describe the *SpTRK* gene that encodes a protein of 833 amino acids. The predicted structure contains 12 putative membrane-spanning domains and resembles various high- and low-affinity systems for K^+ transport in yeasts and plants. TKHp, the product of *SpTRK* exhibits high homology to TRK1 and TRK2 of *Saccharomyces cerevisiae* as well as to HKT1 of *Triticum aestivum,* but is not related to HAK1 of another ascomycete, *Schwanniomyces occidentalis,* suggesting that different routes for potassium uptake evolved independently. This protein is a potassium-specific transporter since functional analysis of the *SpTRK* complemented mutant strain of *Sacch. cerevisiae* revealed potassium transport affinities and uptake characteristics similar to those obtained in wild-type *Sch. pombe.* Patch-clamp analysis in the whole-cell mode confirmed the TKHp-mediated inward current in the complemented strain. The inward current increased by acidification of the extracellular medium thereby suggesting a mechanism of K^+H^+ cotransport. The inward current is not detectable when external K^+ is substituted by Na^+, documenting a distinct cation specificity of the protein.

Key words: K^+ uptake system — Genetic complementation — Transport kinetics — Patch-clamp analysis — Ion-selectivity — *Schizosaccharomyces pombe*

Introduction

Potassium is the most abundant cation in living cells. The transport of K^+ across the plasma membrane regulates the membrane potential and is connected with osmoregulation, transport, nutrition and diverse other cell growth and development processes. In prokaryotes, several different K^+ transport systems have been identified (Bakker, 1992). In higher eukaryotes the cellular K^+ uptake is accomplished by the Na^+/K^+-ATPase (Kawakami et al., 1985; Shull et al., 1986; Modyanov et al., 1991) and inward-rectifying K^+ channels (Ho et al., 1993; Kubo et al., 1993; Schroeder et al., 1994). In fungi and plants an H^+-pumping ATPase is the primary source of energy for the transport of many nutrients (Serrano, 1989; Goffeau & Slayman, 1981). In response to the membrane potential generated by the H^+-ATPase, K^+ uptake can be mediated by inward-directed K^+ channels. Cloning and functional characterization of cDNAs that encode plant inward-rectifying K^+ channels (Anderson et al., 1992; Schachtmann et al., 1992; Sentenac et al., 1992; Bertl et al., 1995; Müller-Röber et al., 1995) showed that they belong to the general class of channels that are activated by membrane hyperpolarization, thereby allowing K^+ influx along its electrochemical gradient. Those K^+ inward-rectifying channels have to be distinguished from K^+ uptake transporters that accomplish potassium uptake coupled to the movement of other cations, as has been postulated for the HKT1 encoded high-affinity K^+ transporter of *Triticum aestivum* (Schachtman & Schroeder, 1994). In the budding yeast *Saccharomyces cerevisiae* two distinct gene products display dual affinities for K^+ transport (Rodriguez-Navarro & Ramos, 1984; Gaber et al., 1988; Ko et al., 1990) and have been suggested to provide the major pathway for potassium uptake into the cell. In contrast, in the ascomycete *Schwanniomyces occidentalis* low- and high-affinity uptake is likely to be mediated by only one gene product, HAK1 (Banuelos et al., 1995).

Schizosaccharomyces pombe contains two plasma membrane H^+-ATPases (Ghislain et al., 1988; Ghislain

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& Goffeau, 1991), at least one species of K^+ channel (Vacata et al., 1992) and a kinetically identified transport system that mediates the uptake of potassium with a K_T of 0.4 mM and a V_{max} of 16 nmol K^+ min^{-1} mg^{-1} dry wt (Rothe & Höfer, 1994). The cytosolic K^+ concentration in *Sch. pombe* is maintained within the range of approximately 175–300 mM (Rothe & Höfer, 1994). We report here the isolation and functional characterization of a cDNA, corresponding to the previously identified *SpTRK* (Soldatenkov et al., 1995), from the fission yeast *Schizosaccharomyces pombe* that encodes a potassium-specific transport protein. We propose a K^+ uptake mechanism coupled to the cotransport of H^+ driven by the $\Delta\mu_H^+$ generated by the H^+-ATPase (Goffeau et al., 1989; Borst-Pauwels, 1993). Our results suggest that the *SpTRK* gene encodes the major K^+ uptake system of *Sch. pombe*. This K^+ transporter shows similarity to the *Saccharomyces cerevisiae* transporters TRK1 and TRK2, to the high-affinity potassium transporter (HKT1) of *Triticum aestivum* (Schachtmann & Schroeder, 1994) and to a lower degree of the *Schwanniomyces occidentalis* transporter HAK1 (Banuelos et al., 1995).

Materials and Methods

Strains, Media and General Genetic and Molecular Methods

All yeast strains were maintained on SDAP medium (Rodriguez-Navarro & Ramos, 1984) containing either 50 mM KCl or 1 mM KCl. The pH was adjusted to 6.5 by H_3PO_4 unless otherwise stated. The following *Sacch. cerevisiae* strains constructed for this study were isogenic derivatives of strain PC1 *trk1 ura3*Δ (Ramos et al., 1985; Sentenac et al., 1992): JRY337, *MATa trk1, trk2*Δ*1::hisG ura3*Δ; and JRY339, *MATa trk1::ura3*Δ*456 trk2*Δ*1::hisG ura3*Δ. Strain S288c *MAT*α *mal mel gal2* was obtained from the Yeast Genetic Stock Center (U.C. Berkeley). To perform one-step gene disruption (Rothstein, 1991) of the *TRK2* locus, a 1529 bp fragment of the *TRK2* gene was first amplified from *Sacch. cerevisiae* genomic DNA by PCR with Taq DNA Polymerase (Serva) using primers corresponding to nucleotides 1328–1352 (5′ GAAAGGTACTTCGACAACATTAGGG 3′) and 2823–2849 (5′ TTCTTGTGCATACAGACTGGAAGAGGC 3′) of the published sequence (Ko & Gaber, 1991). The EcoRV/SacI treated PCR product was gel purified and ligated with purified EcoRV/SacI digested pBluescript II KS (Promega). Plasmid pBS311-6, recovered from transformed *E. coli* DH5α cells, was restriction mapped and partially DNA sequenced. A deletion allele, *trk2*Δ*1::hisG-URA3-hisG*, was created by insertion of the blunt-ended 3.8 kb Bg1II/BamHI fragment containing the *hisG-URA3-hisG* construct from pNKY51 (Alani et al., 1987) into the blunt-ended XbaI/BstXI site of pBS311-6 to generate plasmid pBS315-9. The 4.6 kb SalI/SacI fragment containing *trk2*Δ*1::hisG-URA3-hisG* from pBS315-6 was introduced into PC1 *ura3*Δ cells by DNA mediated transformation followed by selection for uracil prototophy on SD agar medium supplemented with 50 mM KCl. Transplacement of the *TRK2* locus by the *trk2*Δ*1::hisG-URA3-hisG* mutation was confirmed by hybridization analysis. Following excision of the URA3 marker by mitotic recombination between *hisG* repeats, a ura^- derivative, JRY337, was obtained by patching onto 5-FOA plates (Alani et al., 1987). Strain JRY337 required medium supplemented with 50 mM KCl for normal growth. The *trk1::ura3*Δ mutation was created by insertional inactivation of the *TRK1* gene. Plasmid YcP5011-F8 containing the *TRK1* gene was isolated by hybridizing ^{32}P radiolabeled oligonucleotides corresponding to the 5′ and 3′ ends of the published sequence for the *TRK1* coding sequence (Gaber et al., 1988): TRK1-5: 5′ ATAACTAACAATGCATTTTAGAAGAACG 3′, TRK1-3: 5′ TGAGTATATATGTTAGAGCGTTGTGCTGC 3′ to HYBOND membrane (Amersham) replicas of *E. coli* colonies containing a yeast genomic library (Rose et al., 1987). The 2.4 kb XbaI/KpnI fragment of the *TRK1* gene from YcP011-F8 was gel purified and ligated with purified Xbal/KpnI digested pBluescript II KS (Promega). A disrupted allele, *trk1::URA3* was created by insertion of the *URA3* gene into the PstI site. The resulting plasmid (pBS576) was determined by DNA sequencing to contain the *URA3* gene in an opposite orientation to *TRK1*. The 3.4 kb Xbal/KpnI fragment containing *trk1::URA3* was released from pBS576 and introduced into JRY337 cells by DNA mediated transformation followed by selection for uracil prototrophy on SD agar medium supplemented with 50 mM KCl. Transplacement of the *TRK1* locus by the *trka1::URA3* mutation was confirmed by hybridization analysis. To inactivate the *URA3* gene, a nonfunctional allele, *ura3*Δ*456*, was created by removing the 456 bp PstI/StuI internal fragment of the *URA3* gene that had previously been cloned as a blunt-ended HindIII fragment from pGT5 into the EcoRV/SmaI site of pBluescript II KS. The 733 bp XbaI/KpnI fragment containing the *ura3*Δ*456* allele was introduced into the *trk1::URA3* strain by DNA mediated transformation followed by selection for growth on 5-FOA medium to generate strain JRY339. The *Sch. pombe* cDNA library was obtained from F. Lacroute (CNRS, Gif sur Yvette, France). The yeast library was constructed in the expression vector pFL61 as described in Minet et al. (1992), a vector containing the promoter of the phosphoglycerate kinase gene (Kingsman et al., 1990; Bonneaud et al., 1991). The library represents about 1.3×10^7 independent clones. As judged by restriction analysis from a random sample of *E. coli* transformants, 40% of the inserts are >1 kb and 5% are >2.5 kb (F. Lacroute, *personal communication*).

Growth Rate Assessment

Growth in liquid culture was monitored by cell density at 2 hr intervals by measuring OD at 600 nm and by counting the cells in a Thoma chamber.

K^+ Uptake

Cells were grown to a density of 6×10^7/ml in SDAP medium under selective conditions, harvested, washed several times with double-distilled water and starved for 3 to 4 hr at a density of 1.8×10^8 in 1 mM MES (pH 5). Starved cells were harvested by centrifugation, washed twice in double-distilled water, suspended at a density of 6×10^9 cells/ml and kept on ice. A 0.2 ml aliquot of the cell suspension was taken for each uptake assay and diluted to a final volume of 10 ml in 1 mM MES (pH 5), KCl as stated and 200 mM glucose. The glucose was added last, and the K^+ concentration was monitored by a potassium-specific electrode (Orion 931900) with constant agitation of the cell suspension for 10 to 15 min at 25°C. H^+ extrusion was calculated after titration with 10 mM NaOH to readjust the starting pH.

Internal pH Monitoring

Changes in internal pH were followed by recording the fluorescence values of pyranine introduced into the cells by electroporation (2300 V, 200Ω, 25 μF) according to Pena et al. (1995).

Protoplast Preparation

Cells were harvested by centrifugation (500 g, 3 min), resuspended in 5 ml buffer A (50 mM KH_2PO_4, 40 mM mercaptoethanol, pH 7.2 adjusted with KOH) and then centrifuged again. The pellet was resuspended in 3 ml buffer A and incubated in a waterbath at 30°C for 30 min. Afterwards, 3 ml buffer B (50 mM KH_2PO_4, 40 mM mercaptoethanol, 2.4 M sorbitol, 2 mg/ml zymolyase, 2 mg/ml glucuronidase, 30 mg/ml BSA, pH 7.2 adjusted with KOH was added and the mixture was incubated for 45 min at 30°C. Protoplasts were harvested by centrifugation (500 g, 5 min), resuspended in 5 ml buffer C_A (250 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM MES, pH 7.2 adjusted with Tris) and centrifuged again. The resulting pellet was resuspended in buffer C_B ($= C_A$ + 1% glucose) and put on ice. This procedure yielded protoplasts 5–10 μm in diameter for *Sacch. cerevisiae* wild-type cells, and protoplasts 3–5 μm in diameter for JRY339 and SP827 cells.

Patch Clamping

Protoplasts were transferred into a bath solution containing (in mM) either 150, 175, or 200 KCl along with 5 $MgCl_2$, 10 $CaCl_2$ (pH 7.5 or pH 6.5 or pH 5.5 adjusted with 100 Tris/MES). The selectivity measurements were performed with 150 RbCl, CsCl or NaCl replacing KCl in the bath solution. The pipette solution contained (in mM): 175 KCl, 5 $MgCl_2$, 4 ATP, 0.14 $CaCl_2$, 1 EGTA (pH 7.0 adjusted with KOH). Seals of resistances between 1 and 10 GΩ were obtained using micropipettes of resistances between 3–5 MΩ. The whole-cell configuration was achieved either directly during the seal formation or subsequently through suction or voltage pulses (600 mV, 25 msec). Whole-cell measurements were carried out using instrumentation described previously (Vacata et al., 1992). Currents were recorded in response to 2.7 sec voltage steps progressing from +100 to −180 mV in 20 mV decrements, with a 300 msec holding interval at −40 mV between each pair. The data were filtered at 1,000 Hz. According to the standard electrophysiological sign convention, cation flow from the cytoplasmic side of the plasma membrane to the extracellular side was designated outward current and drawn positive.

Computational Analysis

The *SpTRK* open reading frame was used as query for BLAST (Altschul et al., 1990) and FASTA searches (Pearson & Lipman, 1988; Pearson, 1990). Each retained protein was submitted to binary comparison analysis of homology using the PRSS program of the FASTA (Pearson & Lipman, 1988; Pearson, 1990) software package version 1.7. For each comparison 100 shuffles were done. The P-values calculated by the PRSS program which are based on extreme value distribution instead of normal distribution (Altschul et al., 1994) were compiled for all binary comparisons. A threshold of P-value of 10^{-9} corresponds to more than 19% identity over the complete sequence compared. A phylogenetic tree was obtained by the PHYLIP program (Joe Felsenstein, University of Washington, joe@ genetics.washington.edu) from the performed BLAST searches. The multiple alignment program PILEUP (GCG package version 8.0) was used for the alignment of the amino acid sequences and for identification of characteristic signature sequence motifs. The PALIGN program (PCGENE, Intelligenetics) was used for pairwise alignment of amino acid sequences.

Results

Cloning of *SpTRK* cDNAs

To isolate *Sch. pombe* genes involved in potassium transport the *Sacch. cerevisiae* mutant JRY399 (*trk1::ura3*Δ, *trk2*Δ*1::hisG*), which is deficient for K^+ uptake and thus unable to grow in a low potassium medium, was transformed with a *Sch. pombe* cDNA library (Minet et al., 1992). From approximately 1×10^5 transformants, 15 colonies were selected by growth on low K^+ medium (1 mM KCl-SDAP). The plasmids were recovered from yeast cells and reintroduced into the *trk1 trk2* strain. Four of these transformants showed growth rates comparable to the wild-type strain (Fig. 1), thus indicating that suppression of the mutant phenotype was plasmid dependent. The four plasmids (pSP827, pSP2064, pSP2168, pSP2169) contained overlapping cDNA inserts with different lengths of untranslated regions corresponding to a gene, called *SpTRK*, which was recently reported (Soldatenkov et al., 1995). The cDNA sequence encoded a hypothetical protein of 833 amino acids with a predicted M_r of 94845, designated TKHp (transporter of $\underline{K}^+$ coupled to $\underline{H}^+$). In Southern blot analysis of *Sch. pombe* genomic DNA probed with two different fragments (674 bp BstXI/XhoI; 296 bp XbaI/XhoI) of *SpTRK* under high and low stringency, the hybridization signals indicated no gene duplication but rather a single gene.

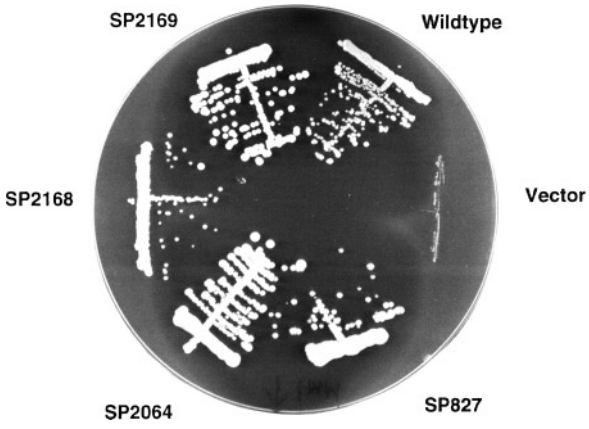

Fig. 1. Complementation of K^+ transport defective *Sacch. cerevisiae* cells by *Sch. pombe* TKHp. Wildtype: *Sacch. cerevisiae* strain S288C was used as wild type *TRK1 TRK2* control in this experiment. Vector: JRY339 cells containing the pPGK vector (pFl61), and SP827, SP2064, SP2168 and SP2169 containing *SpTRK* cDNAs in pF161. The strains were streaked on SDAP medium supplemented with 1 mM KCl and grown for 4 days at 30°C.

SpTRK Hypothetical Protein Product

A hydropathy plot (Kyte & Doolittle, 1982; Fig. 2*A*) indicated twelve putative hydrophobic segments linked by short hydrophilic regions, except for a presumed extracellular loop between TM3 and TM4. Nine of these were predicted to be membrane-spanning segments according to Klein et al. (1985). The 12-segment transmembrane structure is found in a variety of transport proteins belonging to the major facilitator superfamily

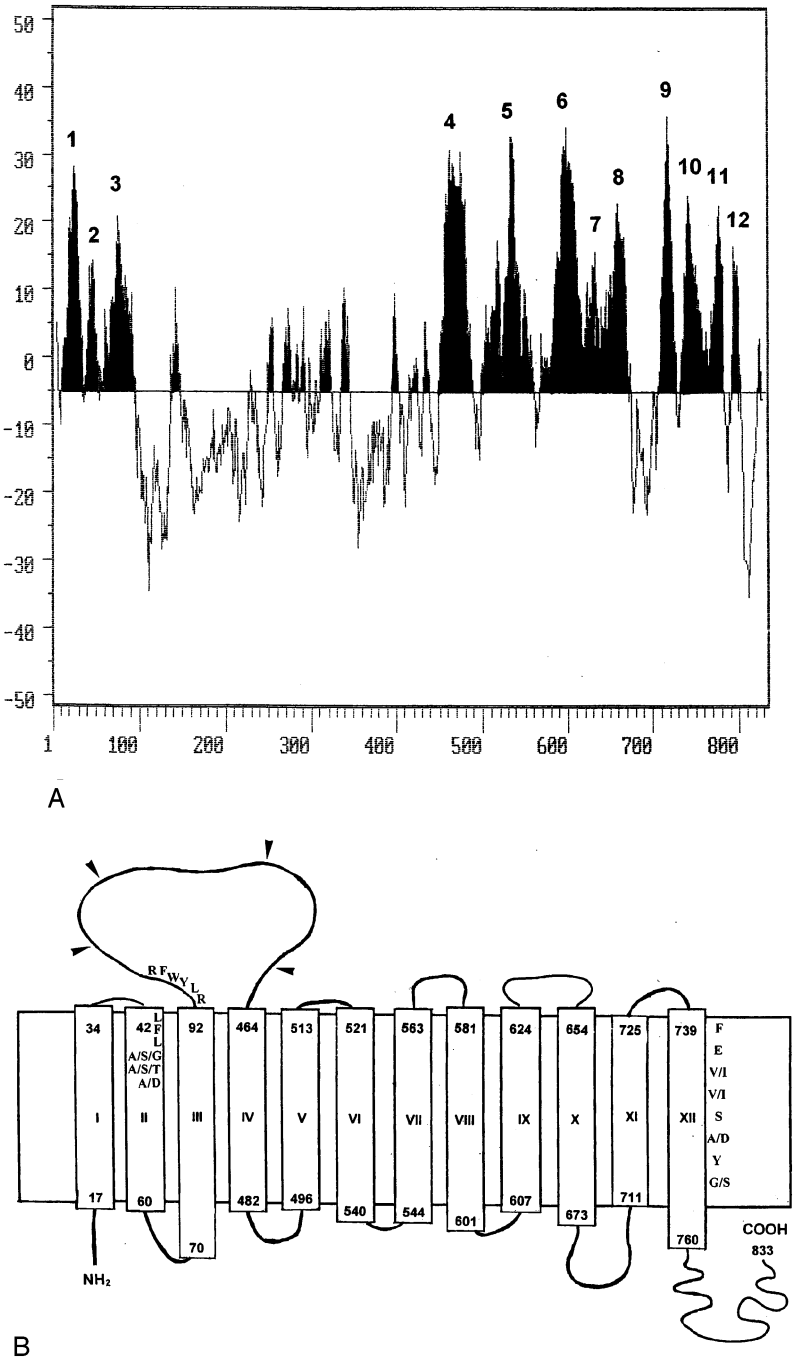

Fig. 2. Hydropathy plot and hypothetical transmembrane structure of the *SpTRK* gene product. (*A*) Average hydropathicity values were determined for spans of 11 residues using the method of Kyte and Doolittle (1982). (*B*) Hypothetical secondary structure model of the TKHp protein. The model is based on the prediction of the hydropathy profile. The putative transmembrane segments are boxed and the first and the last amino acids of each segment are indicated. The most conserved sequence signatures for the aligned potassium transporters (Fig. 3) are indicated to the putative transmembrane topology.

MFS (Marger & Saier, 1993). There are four putative locations of N-linked potential glycosylation sites at positions 108, 156, 207 and 393 (Fig. 2*B*). All of them are situated within the large extracellular loop between the putative transmembrane regions 3 and 4, a feature which is also found in TRK1 and TRK2. Within the 833 amino acid hypothetical protein there was no ATP binding site as seen in various transporters (Gaber et al., 1988; Goffeau et al., 1989; Higgins et al., 1990). The sequence displayed considerable sequence identity (38%) to TRK1 (Gaber et al., 1988) and TRK2 (37%) (Ko and Gaber, 1991) and moderate identity (23%) to HKT1, the high-affinity potassium transporter of *Triticum aestivum* (Schachtmann & Schroeder, 1994). The alignment required the insertion of a substantial number of gaps for HKT1 and included only the putative transmembrane domains for all proteins (Fig. 3). Sequence identity is highest within transmembrane segments TM6–TM12. In particular, the L^{45} FL A/S/G A/S/T A/D^{51} consensus sequence is conserved in putative TM2 and the F^{744} E

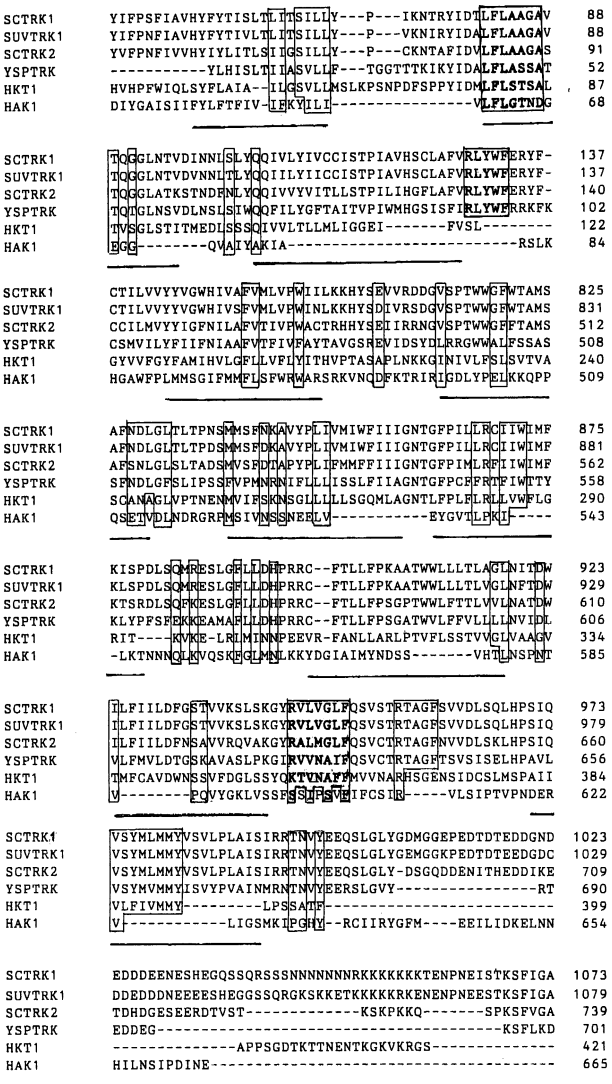

Fig. 3. Sequence alignment for *Sch. pombe* YSPTRK, designated TKHp in this paper, *Sacch. cerevisiae* TRK1 and TRK2, *Sacch. uvarum* TRK1, *Schwanniomyces occidentalis* HAK1 and *Triticum aestivum* HKT1. The PILEUP program (GCG package, version 8.0) was used to align the sequences and for identification of characteristic signature sequence motifs. In this alignment residues which are 80% identical were boxed. The putative transmembrane segments (Fig. 2) are underlined.

V/I V/I S A/D Y G/S^{751} motif is conserved in putative TM12. The R^{93} LYWFR^{99} region, forming a motif at the beginning of the large extracellular loop after putative TM3 aligns within the *Sacch. cerevisiae*, *Sacch. uvarum* (Anderson et al., 1991) and *Sch. pombe* yeast sequences. These conserved features suggest the existence of a K^+ transporter gene family in plants and fungi that appears to have evolved from a common ancester.

Tree Construction

The degree of relatedness between the K^+ transporter proteins was estimated by binary comparisons of all protein sequences from 6 putative transporters in two families. The PRSS software was used which quantifies the identity of two protein sequences by P-values (Table). When the *P*-value is lower than 10^{-8}, the two proteins are considered homologous and belong to the same family (Karlin & Altschul, 1993). One family contains the yeast members TRK1 (*Saccharomyces uvarum*), TRK1 and TRK2 (*Saccharomyces cerevisiae*) with known potassium transporting function and the *Schizosaccharomyces pombe* encoded TKHp. The K^+ transporter HKT1, the only one so far identified in higher plants falls in the same family since the *P*-values for comparison of HKT1 and all other members of the K^+ transporter family is lower than 10^{-14}. In contrast, the *Schwanniomyces occidentalis* encoded HAK1 does clearly not belong to the same family (*P*-values of the order of 10^{-1}). The unrooted phylogenetic tree (Fig. 4) obtained by the PHYLIP program confirms that the K^+ transporter HAK1 from *Schwanniomyces occidentalis* clusters with the Kup transporter from *E. coli* and does not belong to the family made by the other yeast and plant K^+ transporter. The tree also identifies two clusters: One of them contains only the plant transporter HKT1 whereas the TRK proteins from *Sacch. cerevisiae, Sacch. uvarum* and TKH from *Sch. pombe* are closely related.

Table 1. Table of binary comparison scores for the pairwise evolutionary distances between the aligned amino acid sequences

	TKHp	SCTRK1	SCTRK2	SUVTRK1	HKT1
SCTRK1	70				
	38%/13%				
SCTRK2	68	118			
	37%/12%	54%/10%			
SUVTRK1	67	297	132		
	37%/13%	79%/5%	53%/9%		
HKT1	15	15	14	18	
	23%/14%	28%/13%	27%/7%	26%/3%	
HAK1	0.32	0.54	0.24	0.71	0.03
	15%/7%	17%/6%	17%/8%	19%/6%	21%/9%

These proteins all possess between 9 and 12 predicted transmembrane (alpha) helical segments using the KKD algorithm (Klein et al., 1985). TRK1, 1235 aa, 9 spans, Chr X; TRK2, 889 aa, 9 spans, Chr. XI; TKHp, 833 aa, 9 spans; SUVTRK1, 1241 aa, 9 spans; HAK1, 762 aa, 11 spans; HKT1, 533 aa, 9 spans.

On the first line of each row, the negative decimal logarithm of the *P*-value (e.g., 10 means that the *P*-value is 10E-10) is given first (PRSS program). The percent of identity, followed by percent similarity over the entire compared sequence are given in the second line of each row (PALIGN program).

Kinetics of K^+ Transport

The transport properties of the *trk1 trk2* mutant strain complemented with *SpTRK* were analysed by using a K^+ specific electrode measuring extracellular K^+. The cells

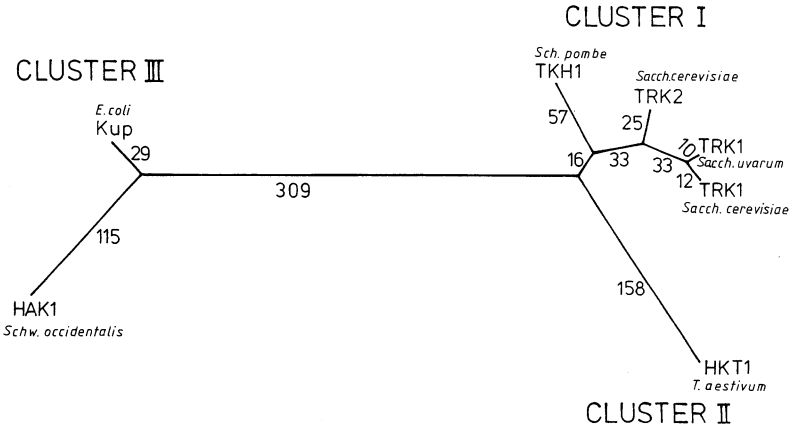

Fig. 4. Unrooted phylogenetic tree. Construction of an unrooted phylogenetic tree obtained by the PHYLIP program for the seven proteins. The proteins depicted are localized to four branches where the encompassed proteins from *Saccharomyces cerevisiae, Saccharomyces uvarum* and *Schizosaccharomyces pombe* origin form cluster I, while the protein from the yeast *Schwanniomyces occidentalis* (HAK1) clusters with the Kup transporter of *E. coli* showing more extensive sequence divergence. The higher plant *Triticum aestivum* (HKT1) K^+ transporter forms a different cluster, but is related to Cluster I.

were incubated in different external K^+ concentrations and K^+ uptake assay was started by the addition of glucose to a final concentration of 20 mM (Fig. 5*A*). There was no net K^+ uptake before glucose was added. The mutant JRY339 (negative control) does not take up K^+ at the indicated low external K^+ concentrations. This is in accordance with the reported half-saturation constant for Rb^+ uptake of 65 mM in a *trk1 trk2* strain (Ramos et al., 1994). In contrast, high rates of K^+ uptake were measured in the strain expressing *SpTRK*. As indicated in the Woolf-Hofstee plot shown in Fig. 5*B*, the kinetics of K^+ uptake in cells expressing *SpTRK* are characterized by a K_T of 0.8 mM and a V_{max} of 15 nmol K^+ min^{-1} mg^{-1} dry wt. These values correspond to those assessed in wild type *Sch. pombe*, K_T 0.4 mM, V_{max} 16 nmol K^+ min^{-1} mg^{-1} dry wt (Rothe & Höfer, 1994). This provides evidence that *SpTRK* cDNA encodes the major potassium transporter of *Sch. pombe*. The above kinetic parameters are different from the values of K_T 0.02 mM and 2 mM reported for the K^+ transporters TRK1 and TRK2, respectively, (Rodriguez-Navarro & Ramos, 1984; Gaber et al., 1988). However, the half-saturation constant of the TRK2 mediated K^+ uptake was recently corrected (Ramos et al., 1994) and coincides now with that of *Sch. pombe* TKHp.

K^+-Dependent H^+ Efflux

To further characterize the uptake mechanism of TKHp the stimulation of H^+ efflux by K^+ uptake was measured by extracellular acidification using a pH electrode and by intracellular alkalinization using pyranine fluorescence quenching (Pena et al., 1995). As shown in Fig. 6, the calculated H^+ extrusion was 950 and 1628 nmol H^+ min^{-1} mg^{-1} fresh wt at external K^+ concentration of 0.1 and 0.3 mM, respectively. Thus the increased potassium uptake is accompanied by an enhanced H^+ extrusion and a corresponding intracellular alkalinization, reflecting the enhanced H^+-ATPase activity with increasing external K^+ concentrations. To assess whether K^+ uptake in *Sch. pombe* is directly dependent on the H^+-ATPase activity (Ghislain et al., 1988) the uptake of potassium was measured in *Sch. pombe* cells in the presence of DCCD, an inhibitor of *Sch. pombe* H^+-ATPase (Dufour & Goffeau, 1980). The inhibition by DCCD of the H^+-ATPase at external pH of 6.3 resulted in no measurable potassium uptake (*data not shown*), supporting the hypothesis that TKHp function is coupled to $\Delta\mu_H^+$ generated by the H^+-ATPase.

Effects of pH and Extracellular K^+ Concentrations

Wild-type cells of *Sch. pombe* could grow over a wide range of external pH from pH 3.5–7.0 with an optimum at pH 4.5. At this pH a decrease in external potassium concentration (7 mM, 1 mM, 0.2 mM) induces a proportional extension of the lag-phase preceding the log-phase of cell growth (Fig. 7*A*). The *trk1 trk2* mutant JRY339 is unable to grow at any of these K^+ concentrations (Fig. 7*B*) whereas the *SpTRK* expressing strain grown at pH 4.5 and at different external K^+ concentrations regained growth parameters characteristic for the wild type *Sch. pombe* (Fig. 7*B*).

Electrophysiological Characterization

The functional properties of TKHp-mediated K^+ uptake were characterized more directly by patch clamping of protoplasts of *Sacch. cerevisiae*, JRY339 and *SpTRK* expressing yeast cells in the whole-cell mode using symmetric K^+ concentrations (Fig. 8). In all cases a potassium outward current activating at +40 mV, probably mediated by the endogenous K^+ channel described recently (Bertl et al., 1993; Bertl et al., 1995; Ketchum et al., 1995; Reid et al., 1996) could be observed and an inward rectifying current in *Sacch. cerevisiae* wild type (Fig. 8*A* and *D*). The inward current was absent in the

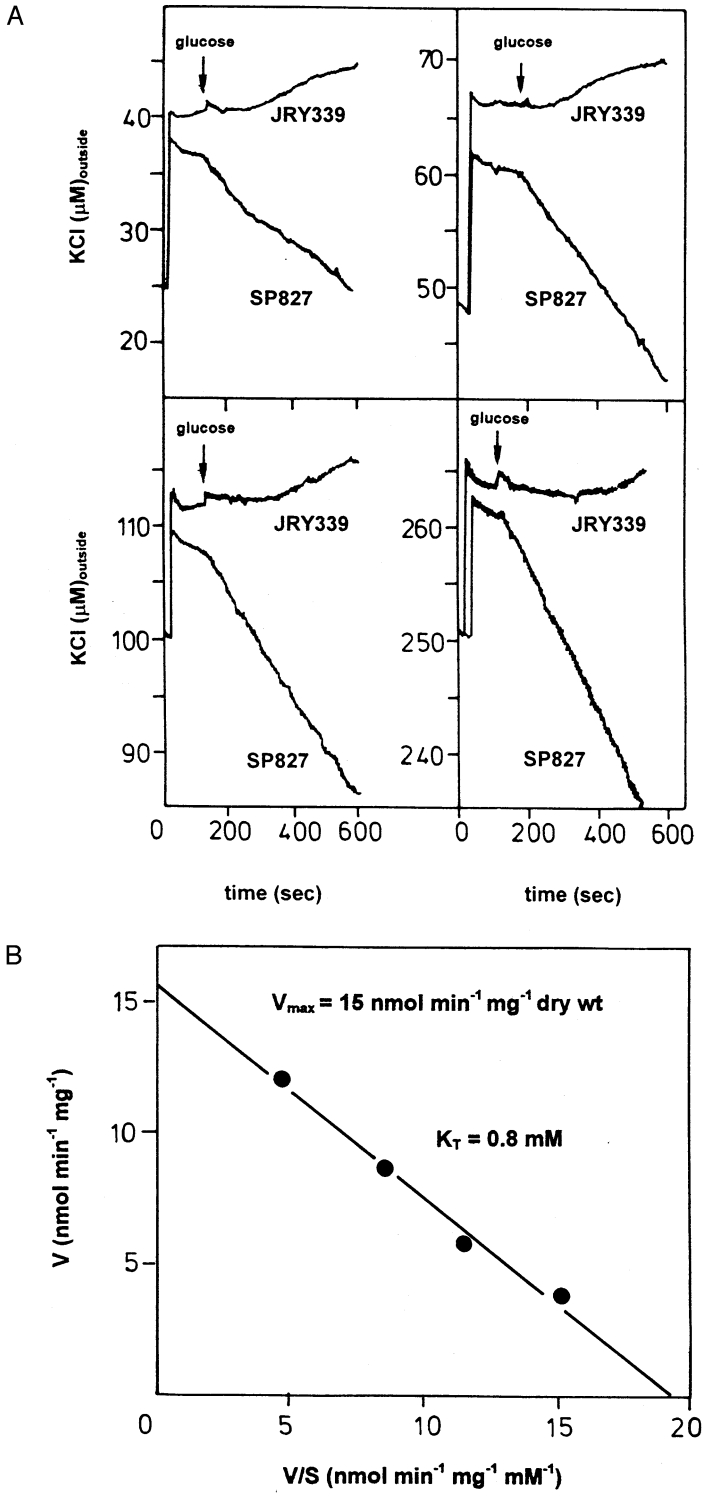

Fig. 5. (*A*) Potassium uptake assays. Depletion of K^+ from the external medium was measured by using a K^+ specific electrode. Assays were performed with *Saccharomyces cerevisiae trk1 trk2* strain JRY339 and *SpTRK* expressing yeast cells (strain SP827) as described in Materials and Methods. The arrows indicate the addition of glucose to start the assay. (*B*) Woolf-Hofstee plot of K^+ uptake in SP827. For the *SpTRK* expressing strain a K_T of 0.8 mM and a V_{max} of 15 nmol K^+ min^{-1} mg^{-1} dry wt was calculated.

trk1 trk2 mutant (Fig. 8*B* and *E*) but was restored in this strain after complementation with *SpTRK* (Fig. 8*C* and *F*). Both voltage dependency and amplitude of *Sacch. cerevisiae* wild-type and TKHp-mediated inward currents are comparable. To define the ion selectivity of TKHp-mediated currents, KCl in the bath solution was replaced by NaCl, RbCl and CsCl (Fig. 9). The inward currents carried by Rb^+ and Cs^+ were slightly smaller than that carried by K^+, indicating a mild selectivity for K^+ over Rb^+ and Cs^+. Substitution of KCl with NaCl

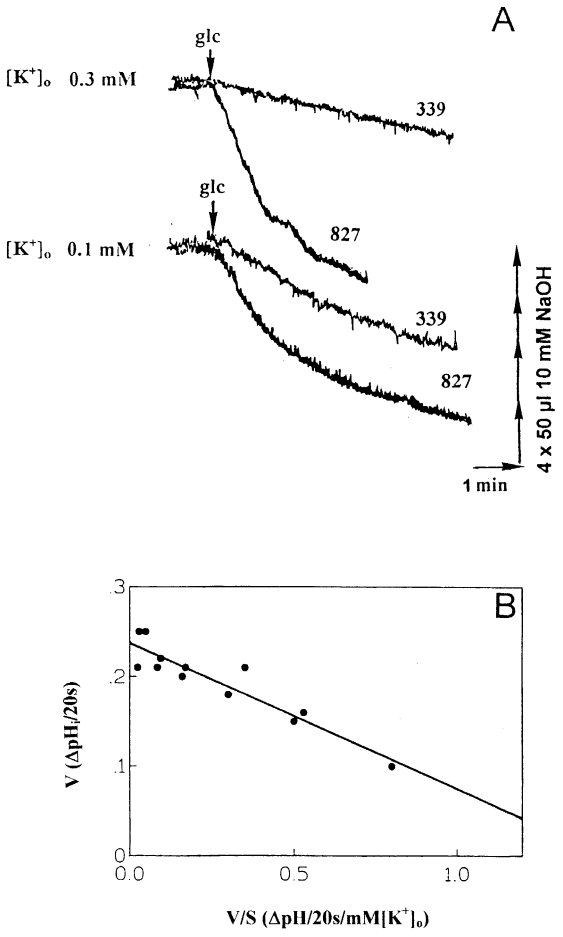

Fig. 6. Intracellular alkalinization caused by K^+ uptake, and concomitant H^+ extrusion induced by the addition of glucose in JRY339 and *SpTRK* expressing strain SP827, starting pH of 6.5. (*A*) At $[K^+]_{out}$ of 0.1 mM and 0.3 mM K^+ extrusions of 950 and 1628 nmol H^+ min^{-1} mg^{-1} fresh wt, respectively, were calculated. (*B*) Woolf-Hofstee plot of intracellular alkalinization caused by K^+ uptake following the addition of glucose.

resulted in no measurable conductance for both the inward and the outward current, reflecting a remarkable discrimination in conductivity. Therefore, a conductance sequence of $K^+ > Rb^+ > Cs^+ \gg Na^+$ is inferred for TKHp. The putative mechanism of proton coupling to K^+ uptake was further investigated by measurements of shifts in the TKHp-mediated conductance. Inward current in *SpTRK* expressing yeast cells was significantly enhanced by acidification of the extracellular medium by two pH units, causing a shift from -328.1 ± 9.1 nA/mm^2 (pH 7.5) to -798.6 ± 28.6 nA/mm^2 (pH 5.5) at -180 mV (Fig. 10), thus supporting the conclusion of proton-coupled K^+ uptake. Further investigations are necessary to prove the significance of the decrease of the endogenous outward current in *Sacch. cerevisiae* at pH 5.5. In addition, whole-cell measurements on protoplasts of wild type *Sch. pombe* are in progress to prove the inward current activation. Nevertheless, the presented measurements show unambiguously that the *Sacch. cerevisiae* mutant JRY339, after complementation with *Sch. pombe SpTRK*, regained its inward rectifying current. These experimental results provide evidence that *SpTRK* cDNA contains the genetic information for the potassium uptake system of *Sch. pombe*.

Discussion

The *SpTRK* cDNA from *Sch. pombe* was cloned by its ability to restore growth of *Sacch. cerevisiae* mutant JRY339 on low potassium. The *SpTRK* cDNA contains an open reading frame encoding a predicted protein of 833 amino acids. Its high degree of hydrophobicity and the presence of twelve transmembrane segments as well as the absence of a putative ATP-binding site place it among integral membrane proteins belonging to the broad class of permeases, symporters and antiporters

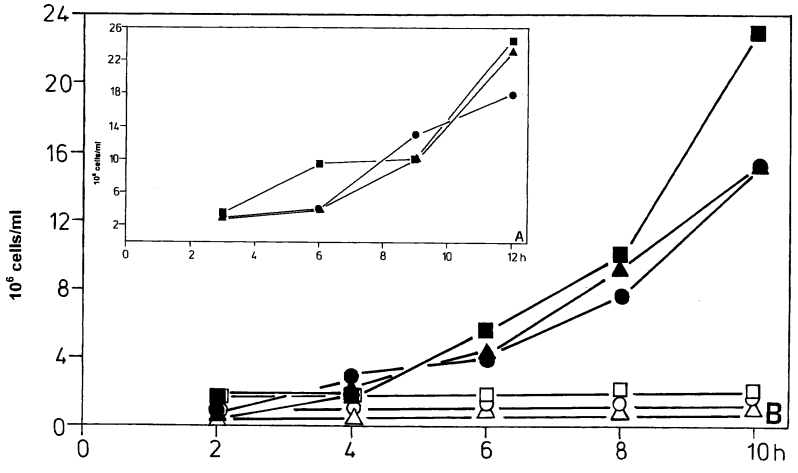

Fig. 7. Effects of extracellular pH and different K^+ concentrations on growth. Growth rate of *Sch. pombe* 972h$^-$ wild type (inset, A), *SpTRK* expressing strain SP827 (*B*, solid symbols) and *trk1 trk2* mutant strain JRY339 (*B*, open symbols) at different external K^+ concentrations. 5×10^7 cells of actively growing cells (preculture of JRY339 in 50 mM KCl-SDAP) were inoculated into liquid SDAP. 0.2 mM K^+ (circles), 1 mM K^+ (triangles) and 7 mM K^+ (squares) at pH 4.5. Growth is expressed as times 10^6 cells per ml.

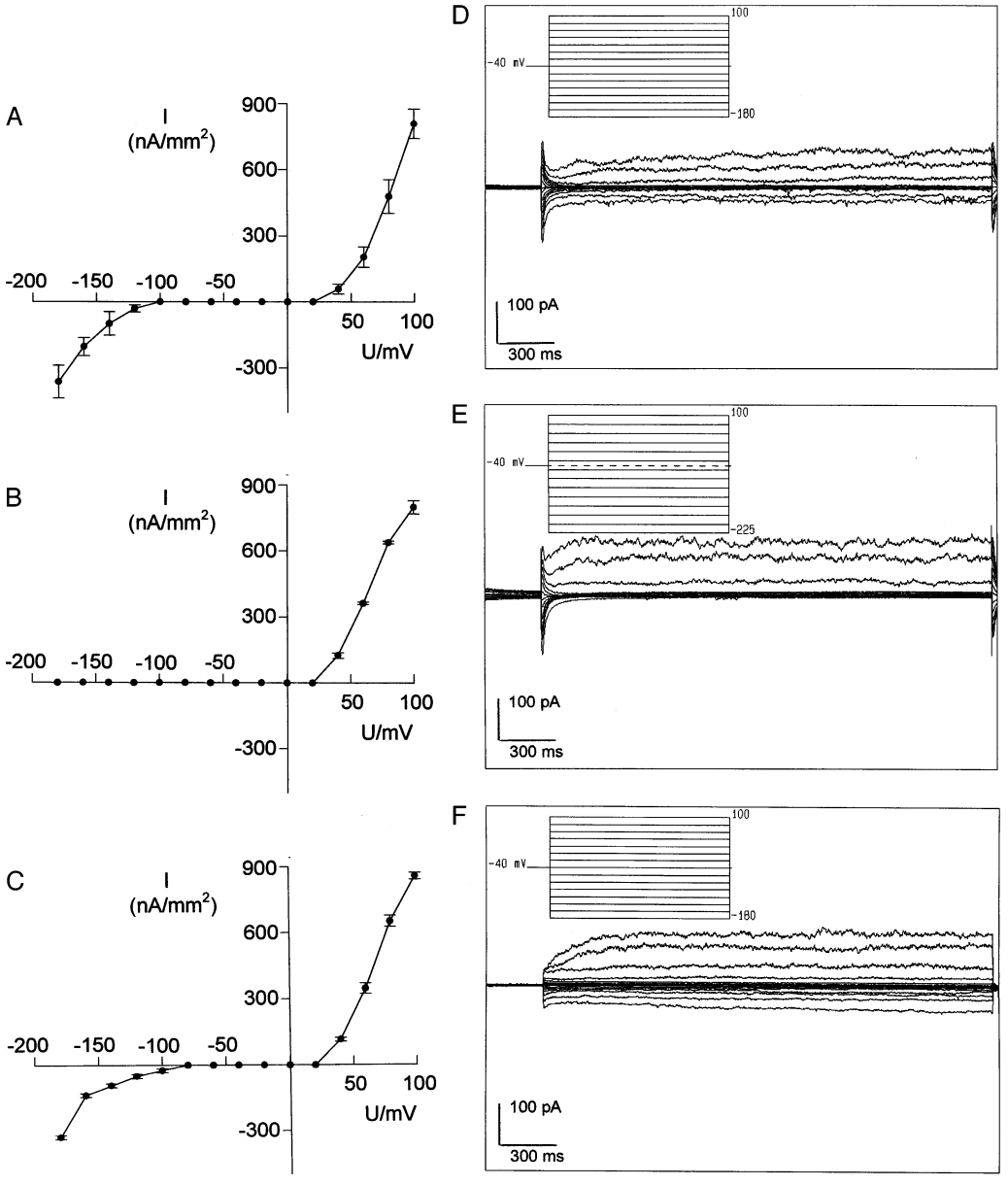

Fig. 8. Patch-clamp measurements in the whole-cell mode of *Sacch. cerevisiae* wild type (*A, D*), *trk1 trk2* mutant strain. JRY339 (B, E) and *SpTRK* expressing strain (*C, F*). The first letter indicates the *I/V* curve and the second letter the corresponding leak subtracted current trace. After seal formation of 1–10 GΩ and obtaining the whole-cell mode configuration, the measurement was started at a holding voltage of −40 mV (300 msec) and subsequent application of voltages in the range of +100 to −180 mV stepwise in 20 mV decrements for 2.7 sec for *Sacch. cerevisiae* wildtype and the *SpTRK* expressing strain. For JRY339 a different voltage protocol was applied in the range of +100 to −225 mV stepwise in 25 mV decrements for 2.7 sec. The resulting *I/V* curves give a potassium outward current activating at +40 mV but no detectable inward current for the *trk1 trk2* mutant and the related control JRY339FL61 (*data not shown*). In contrast, a potassium inward current starting at approximately −100 mV in *Sacch. cerevisiae* wild type (S288C) and in the *SpTRK* expressing strain could be observed.

called MFS (major facilitator superfamily) -*cf.* Marger and Saier (1993). At present we can not exclude the possibility that the topology of 12 transmembrane spans predicted by the Kyte and Doolittle (1982) algorithm has to be modified, since other algorithms evaluated different number of transmembrane spans. Therefore, investigations are in progress to experimentally characterize the TKHp transmembrane topology in detail.

The precise mechanism for high-affinity K^+ uptake in yeast has not been established. Three mechanisms have been proposed, which are based on physiological studies on K^+ uptake in plants and mammals (Schroeder

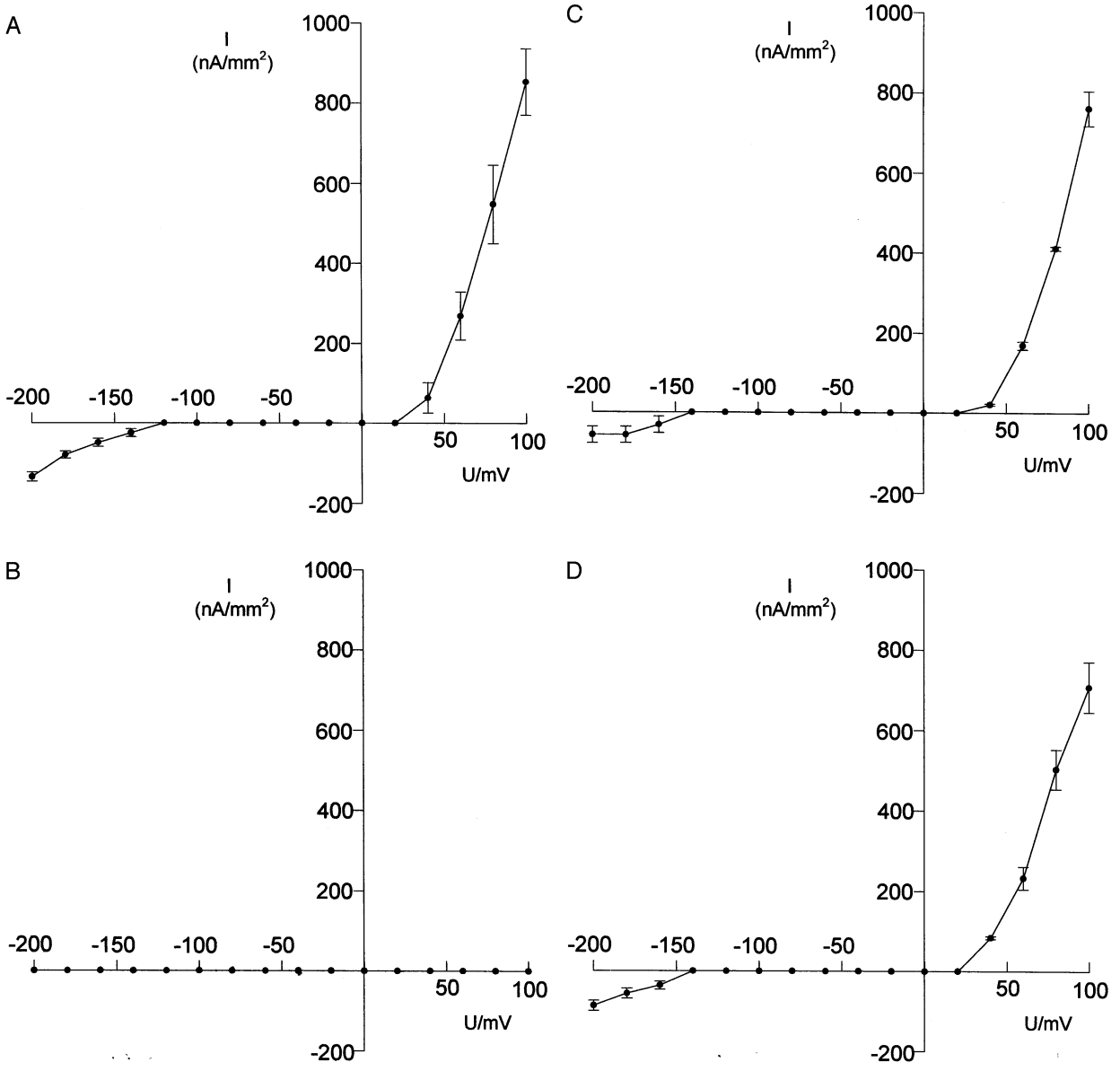

Fig. 9. Ion-selectivity of TKHp. Whole-cell TKHp currents are mildly selective for K^+ over both Rb^+ and Cs^+, but are blocked by NaCl as shown by *I/V* curves in extracellular KCl (*A*), NaCl (*B*), RbCl (*C*) and CsCl (*D*). All cationic concentrations were 150 mM.

et al., 1994; Pedersen & Carafoli, 1988). They include: K^+-H^+ exchange ATPases, K^+ uptake ATPases (Leonard & Hotchkiss, 1976), and K^+-H^+ cotransporters (symporters) that allow influx of both K^+ and H^+ driven by the transmembrane electrochemical proton gradient ($\Delta\mu_H^+$), as found in *Neurospora crassa* (Rodriguez-Navarro et al., 1986) and *Triticum aestivum* (Schachtmann & Schroeder, 1994). We showed that TKHp is a potassium-specific transporter that mediates K^+ uptake with kinetics characteristic of the native transport system in *Sch. pombe*. The K_T values for K^+ uptake in *Sch. pombe* wild type and in *SpTRK* expressing yeast cells, 0.4 mM and 0.8 mM, respectively, are different from those reported for high-affinity K^+ uptake mediated by yeast TRK1 (20 μM, Rodriguez-Navarro & Ramos, 1984) and plant HKT1 (29 μM, Schachtmann and Schroeder, 1994).

The K^+ transporter encoded by *SpTRK* functions to complement the K^+ uptake deficiency in the *Sacch. cerevisiae* mutant strain JRY339 (*trk1 trk2*). Consistently, the rescue coincided with the formation of inward rectifying currents in *SpTRK* expressing cells similar to those of the *Sacch. cerevisiae* plasma membrane. Regarding its electrical properties, TKHp is characterized by an activation potential of −100 mV and remains active even during prolonged hyperpolarization. Further insight into the spatial structure of the K^+-conducting pathway of the transporter was obtained from the cation permeability sequence and current/voltage relations in the presence of

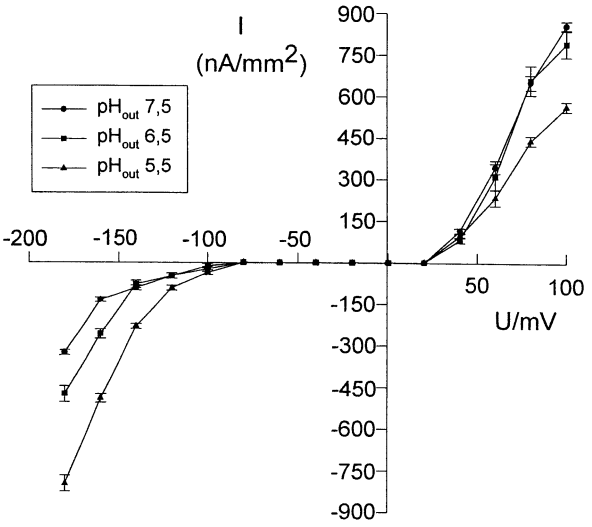

Fig. 10. *I/V* curves of *SpTRK* expressing strain in a bath solution adjusted to pH 7.5, pH 6.5 and pH 5.5. The measurement was started at a holding voltage of −40 mV, with subsequent application of voltages in the range of +100 to −180 mV stepwise in 20 mV decrements for 3 sec. Note the strong increase with each acidification by one pH unit resulting in the more than doubled inward potassium current at pH 5.5.

permeating ions other than K^+. The TKHp protein is conductive for K^+ and also accepts the larger cations, Rb^+ and Cs^+, but seems to be completely blocked by Na^+, thereby displaying high selectivity. The proposed proton-coupled K^+ uptake was indicated by patch-clamp experiments when the extracellular pH was acidified from 7.5 down to pH 5.5 (Fig. 10). The magnitude of the inward current increases more than 2-fold with decreasing external pH by 2 units suggesting a mechanism of proton-coupled K^+ uptake for TKHp in energized cells, driven by $\Delta\mu^+_H$. The pma1$^+$ encoded outward-directed H^+-pump is capable of hyperpolarizing the membrane potential and of acidifying the external medium, thus regulating the activity of TKHp. Taken together, the functional complementation of JRY339 by *SpTRK*, as well as the electrogenic nature of TKHp-mediated transport, the lack of a catalytic ATP-binding domain, and the pH dependency of K^+ uptake suggest that K^+-H^+ symport is the biologically relevant mechanism of K^+ uptake in *Sch. pombe*. K^+/H^+ symport is a thermodynamically active process that would provide a high-affinity K^+ uptake pathway with the capacity to accumulate K^+ from solutions containing 10 μM K^+. K^+/H^+ symport would be an appropriate mechanism for K^+ uptake in fungi and plants that take up K^+ directly from the environment at low concentrations, thereby accumulating high intracellular concentrations required for growth.

Compared with the other K^+ transport proteins, TKHp is most closely related to the sequences from *Sacch. cerevisiae* and *Sacch. uvarum* in terms of the presence of several highly conserved amino acid motifs. The motif present between putative TM3 and TM4, RLYWFR, seems to be characteristic for all yeast K^+ transporters of cluster I (Fig. 4). Other conserved motifs, such as LFL (TM2) and FE V/I V/I S A/D Y (TM12) found also in another ascomycete (*Schwanniomyces occidentalis*) and in higher plant (*Triticum aestivum*), may be related to the general potassium uptake. The functional significance of those motifs has yet to be established. The cloning and functional characterization of TKHp as K^+-uptake transporter enables further detailed molecular, physiological and biophysical studies of K^+ nutrition and transport and of related environmental stresses, such as K^+ deficiency, in *Sch. pombe*.

We thank F. Lacroute (CNRS, Gif-sur-Yvette) for the generous gift of the *Sch. pombe* library. Part of the work was carried out during a research stay of M.H. at the Universidad Nacional Autonomia de Mexico, which was sponsored by Volkswagen-Stifung, Hannover, and CONACyT, Mexico, D.F. This work was supported by the Deutsche Forschungsgemeinschaft, grant No. Ho 555/19-1, and partially by NATO Linkage Grant HTECH.LG 930686.

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