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A Trk/HKT-Type K^+ Transporter from *Trypanosoma brucei*[▽]

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The molecular mechanisms of K^+ homeostasis are only poorly understood for protozoan parasites. *Trypanosoma brucei* subsp. parasites, the causative agents of human sleeping sickness and nagana, are strictly extracellular and need to actively concentrate K^+ from their hosts' body fluids. The *T. brucei* genome contains two putative K^+ channel genes, yet the trypanosomes are insensitive to K^+ antagonists and K^+ channel-blocking agents, and they do not spontaneously depolarize in response to high extracellular K^+ concentrations. However, the trypanosomes are extremely sensitive to K^+ ionophores such as valinomycin. Surprisingly, *T. brucei* possesses a member of the Trk/HKT superfamily of monovalent cation permeases which so far had only been known from bacteria, archaea, fungi, and plants. The protein was named TbHKT1 and functions as a Na^+-independent K^+ transporter when expressed in *Escherichia coli*, *Saccharomyces cerevisiae*, or *Xenopus laevis* oocytes. In trypanosomes, *TbHKT1* is expressed in both the mammalian bloodstream stage and the Tsetse fly midgut stage; however, RNA interference (RNAi)-mediated silencing of *TbHKT1* expression did not produce a growth phenotype in either stage. The presence of *HKT* genes in trypanosomatids adds a further piece to the enigmatic phylogeny of the Trk/HKT superfamily of K^+ transporters. Parsimonial analysis suggests that the transporters were present in the first eukaryotes but subsequently lost in several of the major eukaryotic lineages, in at least four independent events.

Potassium (K^+) is the most abundant cation in the cytosol of any cell and hence an essential macronutrient for life on earth. Concentrative K^+ uptake across the plasma membrane is energized directly by ATPases and indirectly by the negative membrane potential or by coupling, via symport or antiport, to other transport processes such as H^+ flux. The ancestry of K^+ transporters renders them ideal subjects for phylogenetic comparisons. Indeed, the different kinds of known K^+ transporters—pumps, channels, permeases, symporters, and antiporters—are all found in bacteria (43). Eukaryotes do not appear to have invented further mechanisms of K^+ transport; on the contrary, some families of K^+ transporters were lost over the course of eukaryote evolution, particularly among the metazoa (53).

The Trk/HKT superfamily (TC transporter classification 2.A.38 [43]) consists of bacterial TrkH and KtrB, plant HKT, and fungal Trk transporters (15). These proteins share a topology with 8 transmembrane (TM) domains and, sandwiched between odd- and even-numbered TM domains, 4 shorter hydrophobic helices that resemble the P-loops of K^+ channels (14, 27, 55). In the K^+ channel, these pore-forming loops end in the filter residues glycine-tyrosine-glycine, which coordinate K^+ by means of their backbones' carbonyl oxygens (13). The P-loop-like helices of Trk/HKT transporters end in a single conserved glycine (48), and these glycines have been shown to determine K^+ selectivity over Na^+ of the transporters (34, 49). Thus, a Trk/HKT monomer with 8 TM domains and 4 P-loops is thought to have a similar pore architecture to a K^+ channel tetramer with two TM domains and one P-loop per subunit. The Trk/HKT transporters are important for cellular K^+ acquisition in microorganisms, since *trk* null mutant yeast or bacteria exhibit growth phenotypes on media containing low K^+ concentrations (20, 46). The roles of the Trk/HKT transporters in plants are more diverse, including Na^+ distribution (10, 33, 47), osmoregulation (32), and salt tolerance (39). So far, no HKT/Trk transporter has been described from the metazoa or protista.

Trypanosoma brucei subsp. parasites comprise the causative agents of human and livestock trypanosomosis: sleeping sickness and nagana, respectively. The distribution of the parasites is restricted by that of their vector, the blood-sucking tsetse fly (*Glossina* spp.), to the so-called tsetse belt comprising 36 countries between the Sahara desert and the Kalahari (3, 8). African trypanosomes proliferate extracellularly in the blood, evading the mammalian immune response by antigenic variation. Untreated sleeping sickness is fatal. There is an urgent need for new and better drugs since the current ones, the arsenical melarsoprol in particular, suffer from severe side effects (31). In the mammalian bloodstream, the parasites encounter a rich and steady supply of nutrients, readily imported by specific permeases or endocytosed via receptors (7). Research on trypanosomal nutrient uptake has so far concentrated on transporters of organic substrates: nucleobases, nucleosides, sugars, and amino acids (4, 12, 26, 30, 35, 56). Little is known about how the parasites import inorganic nutrients. The malaria parasite *Plasmodium falciparum* possesses two putative K^+ channel subunits with 6 TM domains and one P-loop (19, 52). Disruption of an orthologous gene in *Plasmodium berghei* strongly impaired the development of the malaria

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parasites in the mosquito (18). However, these putative channels have not yet been proven to be permeable to K^+. The *T. brucei* genome (6) is annotated to contain two putative K^+ channels; in addition, a putative ATPase has been identified resembling fungal Na^+/K^+ efflux ATPases (5, 45). None of these has been addressed experimentally. Here we present the identification and characterization of TbHKT1 (Tb10.70.2940), a Trk/HKT-type K^+ transporter from *Trypanosoma brucei* and representative of a new clade of *Trk/HKT* genes from kinetoplastid parasites.

MATERIALS AND METHODS

***In silico* methods.** A Trk/HKT-specific profile (hkt.hmm; available on request) was made with HMMer (17) from a ClustalW multiple alignment (50) of a homology-reduced set of representative Trk/HKT proteins and validated against the proteomes of *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. Parasite predicted proteomes were downloaded from ftp.sanger.ac.uk (*T. b. brucei* v4.0, *Leishmania major*, and *Trypanosoma cruzi*), www.plasmoDB.org (*P. falciparum* v5.5), ftp.ebi.ac.uk/pub/databases/integr8 (*Theileria parva*), ftp.tigr.org (*Entamoeba histolytica* and *Trichomonas vaginalis*), and www.giardiadb.org (*Giardia duodenalis* v1.1). The obtained hits were run through TMHMM (29) to predict membrane topology and searched against the GenBank nonredundant protein database with Blastp (2) to test for eventual relationships to other, non-Trk/HKT sequences. The phylogenetic tree (see Fig. 3) was drawn from a ClustalW alignment with Dendroscope (25). Bootstrapping was performed for 1,000 rounds, and results were expressed as percent positives.

Cell lines and media. Monomorphic bloodstream-form *T. b. brucei* 221 (MiTat 1.2; 221) parasites were cultivated at 37°C, 5% CO_2 in HMI9 medium supplemented with 5% fetal calf serum (24). Procyclic *T. b. brucei* 427 (derived from MiTat 15a) parasites were grown at 27°C in SDM medium supplemented with 5% fetal calf serum (9). For RNA interference (RNAi) experiments, bloodstream-form *T. b. brucei* "New York single-marker" and procyclic *T. b. brucei* 29-13 were used (54). Transfectants were selected in 0.1 mg/ml puromycin and cultivated in the presence of 1 mg/ml tetracycline to induce transcription of the stem-loop RNAi construct (1). The rat L6 myoblast cell line was obtained from ATCC (CRL-1458) and cultivated at 37°C with 5% CO_2 in RPMI 1640 medium supplemented with 2 mM L-glutamine, 5.95 g/liter HEPES, 2 g/liter $NaHCO_3$, and 10% fetal calf serum. Yeast complementation experiments were performed with *trk1*- and *trk2*-deficient *S. cerevisiae* strain CY162 (20) on arginine/phosphate medium (41), consisting of 8 mM phosphoric acid, 10 mM L-arginine, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, 20 mg/liter histidine, 2% galactose, and 0.3% sucrose, supplemented with the standard vitamins and trace elements and 1.5% agarose (because agar contains too much potassium). KCl was added to the desired concentration. *Escherichia coli* complementation was performed with the *trkD* (*kup1*) *trkG trkH kdpABC5* quadruple mutant LB2003 (46) grown in a medium containing 46 mM Na_2HPO_4, 23 mM NaH_2PO_4, 8 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 6 μM $FeSO_4$, 1 mg/liter thiamine, 1% glucose, 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and KCl to the desired concentration.

Drug sensitivity tests. Trypanosomes (10^3 bloodstream forms per well, respectively, 10^4 procyclic forms per well) or L6 myoblasts (4×10^3 per well, seeded 24 h prior to the addition of test compounds) were cultivated in 100 μl medium on a 96-well plate containing serial dilutions of test compounds. After 70 h of incubation, 10 μl per well Alamar Blue solution was added (12.5 mg resazurin in 100 ml phosphate-buffered saline) and after another 2 h of incubation, fluorescence was measured with a Spectramax Gemini fluorimeter (Molecular Devices, Sunnyvale, CA) at 536 nm excitation and 588 nm emission. Fifty percent inhibitory concentrations (IC_{50}s) were determined by nonlinear regression to a sigmoidal dose-response curve with Prism 5 (GraphPad Software, Inc.). Each assay was performed in triplicate and repeated at least 3 times. All chemicals were purchased from Sigma/Fluka.

FACS analysis. Cells (8×10^6 procyclic forms [PCF] or 10^6 bloodstream forms [BSF]) were incubated with 100 mM K^+-glutamate, 1.5 μM valinomycin, or both at 27°C (PCF) or 37°C (BSF) for 20 min. Then the cells were brought to a density of 1×10^6/ml, and 1 min before fluorescence-activated cell sorter (FACS) measurement, 400 nM the anionic $DiBAC_4(3)$ (bis-[1,3-dibutylbarbituric acid] trimethine; Molecular Probes) was added and flow cytometry was performed with a Becton Dickinson FACSCalibur (fluorescence window, 505 nm to 530 nm). The lower the membrane potential is (i.e., the more depolarized), the more the anionic fluorescent DiBAC enters the cell. For each measurement, 10,000 cells were analyzed. Data were evaluated with the CellQuest Pro software version 5.2.

Cloning and plasmid constructs. *TbHKT1* was amplified from genomic DNA of *T. b. brucei* 221 by PCR with the forward primer (GGGATCCACGCGATATGG CCAGTCAA; BamHI site underlined) and the reverse primer (GTCTAGAACTC ACGGCAGCGAATTGA; XbaI site underlined). The product was cloned via the incorporated restriction sites into pYES2 for expression in *S. cerevisiae*, into pPAB404 for expression in *E. coli*, and into pOMU for *in vitro* transcription of cRNA. For RNAi-mediated gene silencing, a 280-bp fragment of TbHKT1 encompassing nucleotides 1489 to 1771 (codons 496 to 590) was amplified by PCR from genomic DNA of *T. b. brucei* with the forward primer GCAAGCTTGGATCCCG TACGCCATCAGACCATTCACCT (HindIII site underlined and BamHI site double underlined) and the reverse primer GCTCTAGACTCGAGTGCTGAAATCC ACAGTATAGTGGCC (XbaI site underlined and XhoI site double underlined), and cloned into pGEM-Teasy (Promega). The insert was subcloned twice, in opposite directions separated by a 460-bp stuffer, into the vector pALC14 (1), a derivate of pLEW100 (54).

Gene expression analyses in trypanosomes. Total RNA was extracted from trypanosomes by the "hot phenol" method. About 10^8 cells were lysed in a preheated (90°C) mixture of 50% phenol, 0.5% SDS, 45 mM NaCl, 4.5 mM Tris-HCl (pH 7.5), 2.3 mM EDTA, and centrifuged. After phenol-chloroform extraction, the RNA was ethanol precipitated, washed, and resuspended in diethyl pyrocarbonate (DEPC)-treated water. Total RNA (10 μg) was electrophoresed on a 1.2% agarose gel and blotted on a Nylon membrane (Roche Diagnostics). The RNA was probed with an α-[^{32}P]dCTP-labeled *TbHKT1* probe (Megaprime DNA labeling system; Amersham) and scanned on a PhosphorImager (Amersham Bioscience).

Electrophysiological recordings of *Xenopus* oocytes. Capped cRNA of *TbHKT1* and *A. thaliana* HKT1 (*AtHKT1*) was prepared *in vitro* with the mMESSAGE mMACHINE kit (Ambion, Austin, TX). *Xenopus laevis* oocytes were prepared as described previously (20, 27, 51). Oocytes were kept at 16°C in Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 mM HEPES-NaOH, pH 7.4]. Ionic currents were recorded 1 day after injection of cRNA (10 ng per oocyte) by two-electrode voltage clamping, in a bath solution containing 6 mM $MgCl_2$, 1.8 mM $CaCl_2$, the indicated concentrations of K^+ and Na^+ (as glutamate salts), 10 mM Mes-1,3-bis(tris[hydroxymethyl]methylamino)propane, pH 5.5, and osmolality adjusted to 240 mosmol/kg with D-mannitol. Voltage clamping and data acquisition were performed with voltage clamp amplifiers (Cornerstone model TEV-200 from Dagan Instruments, Minneapolis, MN; or AxoClamp 2B from Axon Instruments, Foster City CA).

RESULTS AND DISCUSSION

Phenotypic characterization of K^+ homeostasis in *T. brucei*. As a first step toward the characterization of the mechanisms underlying K^+ homeostasis in *Trypanosoma brucei*, we measured their sensitivity to perturbation of [K^+] in the medium and their susceptibility to K^+ antagonists. The assays were performed *in vitro*, using the redox-sensitive dye Alamar blue as an indicator of cell viability (40). Both the mammalian-stage bloodstream forms and the tsetse fly midgut-stage procyclic forms of *T. b. brucei* were tested. The trypanosomes tolerated K^+ concentrations of 100 mM and Na^+ concentrations of 200 mM (Table 1), exceeding by far the physiological levels in human serum: 4.4 mM for K^+ and 142 mM for Na^+ (28). The values in Table 1 include the contributions of the medium, which were 5.8 mM K^+ and about 144 mM Na^+ for procyclic as well as bloodstream-form trypanosomes (the sodium concentration is difficult to estimate since the pH of the medium was adjusted with NaOH). The trypanosomes had millimolar IC_{50}s for Rb^+ and Cs^+, albeit they were still an order of magnitude more sensitive to Rb^+ than the rat myoblast cell line included as a reference (Table 1). Furthermore the trypanosomes were rather insensitive to the K^+ channel blocker tetraethylammonium (TEA^+), the bloodstream forms in particular, and they tolerated the channel-blocking charybdotoxin

TABLE 1. Sensitivity of African trypanosomes to K^+ analogs and antagonists[a]

Analog/antagonist	Sensitivity (IC_{50}) for:		
	T. brucei		Rat L6 myoblasts
	Procyclic forms	Bloodstream forms	
Na^+	240 mM	264 mM	291 mM
K^+	150 mM	154 mM	124 mM
Rb^+	8.5 mM	4.1 mM	82 mM
Cs^+	7.9 mM	14 mM	15 mM
TEA^+	12 mM	70 mM	31 mM
Charybdotoxin	>10 μM	>10 μM	>200 nM
Valinomycin	1.4 nM	170 pM	200 nM
Gramicidin	170 pM	1.0 nM	3.4 mM

[a] The cations were administered as chloride salts. IC_{50}s were determined *in vitro* as described previously (40).

from scorpion venom at the highest applicable dose (Table 1). This apparent robustness of the mechanisms underlying K^+ homeostasis in *T. brucei* contrasted with the trypanosomes' extreme susceptibility to the K^+ ionophores valinomycin and gramicidin (Table 1), the trypanosomes being over 1,000-fold more sensitive than the rat myoblasts. Valinomycin and gramicidin themselves may not be of therapeutic value due to their toxicity. Nevertheless, their trypanocidal potency indicates that K^+ homeostasis in African trypanosomes, in spite of their insensitivity toward K^+ analogs and channel blockers, harbors essential and sensitive drug targets.

Cell viability being a rather crude indicator, we used the fluorescent bis-oxonol dye $DiBAC_4(3)$ to monitor by FACS analysis (fluorescence-aided cell sorting) the immediate effect of externally added K^+ on the membrane potential in trypanosomes. $DiBAC_4(3)$ serves as a qualitative potentiometric probe since the influx of the dye is inversely proportional to the polarization of the target cell. Bloodstream-form *T. brucei* trypanosomes exhibited a higher fluorescence after addition of bis-oxonol (400 nM) than procyclic forms, indicating a less negative membrane potential (Ψ_p) in the bloodstream forms (green curves in Fig. 1). This is in agreement with previous studies reporting a membrane potential of −152 mV for procyclics (11) and −82 mV for bloodstream forms (37). The trypanosomes (bloodstream as well as procyclic forms) did not spontaneously depolarize in the presence of high (100 mM, added in the form of glutamate salt in order to prevent potential artifacts from Cl^- currents) extracellular K^+ concentrations (Fig. 1, pink curves). The effect of exogenous K^+ on Ψ_p in *T. brucei* has been controversial (11, 37). Our results indicate that in *T. brucei*, bloodstream as well as procyclic forms, the plasma membrane is not immediately penetrable for K^+. Addition of the K^+ ionophore valinomycin (1 μM) alone did not affect Ψ_p (Fig. 1, blue curves), indicating that K^+ is at electrochemical equilibrium at physiological external $[K^+]$. Only upon addition of 100 mM K^+ in combination with 1 μM valinomycin, did the cells depolarize within minutes (Fig. 1, orange curves). This was harmful to the cells, and they died after 50 min of incubation (not shown). Thus, although K^+ is in equilibrium at resting membrane potential, there do not seem to be open K^+ channels through which the cation could readily pass the *T. brucei* plasma membrane.

A new clade of *Trk*/*HKT* genes from trypanosomatids. The *T. brucei* genome (6) encodes two putative K^+ channels, Tb09.160.3380 and Tb927.1.4450. A further gene product, Tb927.10.16170, is annotated as a putative K^+ channel in the TriTryp database (TriTrypDB.org), but while it has a predicted K^+ channel tetramerization domain (Pfam profile PF02214), it is not predicted to possess transmembrane domains by TMHMM (29). In order to identify further K^+ transporters from *T. brucei*, we screened the predicted proteome (version 4, 9192 proteins) with hidden Markov model-based profiles for various cation transporter families. The profiles were made with HMMer (16) from homology-reduced sets of representative transporters and validated against the predicted proteomes of *Arabidopsis thaliana* and *S. cerevisiae*. Based on these searches, African trypanosomes do not possess cyclic-nucleotide-gated cation channels, KUP/HAK K^+ permeases (TC 2.A.72) or K^+:H^+ antiporters (TC 2.A.37), transporter families which are ubiquitous in plants (21). The only search that returned a significant hit (E-value of 1.5e−5) from *T. b. brucei* was with the Trk/HKT-specific profile; the identified protein, Tb10.70.2940, was named TbHKT1. The gene product Tb10.70.2940 has recently been renamed to Tb927.10.4300 (TriTrypDB.org). The similarity of TbHKT1 to known Trk/HKT proteins is rather low at the level of primary amino acid sequence; the founding member of the HKT family, TaHKT2;1 from wheat (44), was used as a reference in Table 2. The similarity becomes more evident when comparing hydropathy profiles (Fig. 2). TbHKT1 has an extended hydrophilic N terminus of 100 amino acids without similarity to any known sequence, followed by the typical topology of HKT/Trk proteins: four P-loop like domains, each between two predicted TM domains and each followed by a glycine residue (Fig. 2), the presumed K^+ selectivity filter. *TbHKT1* has orthologues in other trypanosomatids: two almost identical alleles in *T. cruzi*, one in *Trypanosma vivax* and in various *Leishmania* spp. (Table 2);

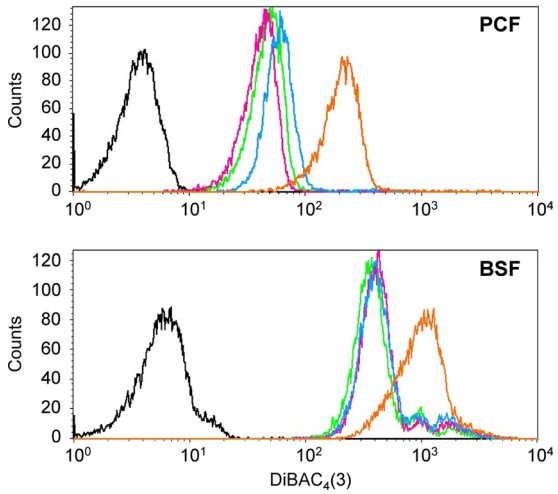

FIG. 1. Trypanosomes do not spontaneously depolarize. *T. b. brucei* procyclic (top) and bloodstream (bottom) forms were incubated with the fluorescent dye DiBAC (colored curves; black curve, untreated control cells, no DiBAC) and the fluorescence was quantified by FACS analysis. Green, normal medium; pink, medium supplemented with 100 mM K^+; blue, medium supplemented with 1 μM valinomycin; orange, medium supplemented with 100 mM K^+ plus 1 μM valinomycin.

TABLE 2. Predicted Trk/HKT proteins from trypanosomatids

Species	Protein	TriTryp accession no.	Length (aa)	% similarity to TaHKT2;1[a]
T. b. brucei	TbHKT1	Tb927.10.4300	599	29
T. vivax	TvHKT1	TvY486_1004280	610	26
T. cruzi	TcHKT1	Tc00.1047053511469.60 Tc00.1047053506295.120	592	33
L. major	LmjHKT1	LmjF35.0080	579	31
L. brasiliensis	LbrHKT1	LbrM34_V2.0120	580	31
L. infantum	LinHKT1	LinJ35_V3.0080	579	30

[a] Percent similarity to TaHKT2;1 from wheat (*Triticum aestivum*) was measured by Needleman-Wunsch global alignments with Blosum62.

only *Trypanosoma congolense* appears to lack a *TbHKT1* orthologue. The respective genomic regions are syntenic in all trypanosomatids (TriTrypDB.org). These trypanosomatid HKTs form a new, distinct branch on the phylogenetic tree of the Trk/HKT superfamily, equally distant to the plant HKT and fungal Trk proteins (Fig. 3). There is no evidence for horizontal transfer of plant *HKT* genes to trypanosomes, as has been suggested for metabolic enzymes (22). In *E. coli* and other bacteria, the K^+ translocator TrkH requires the NAD-binding subunit TrkA (36) and the ATP-binding subunit

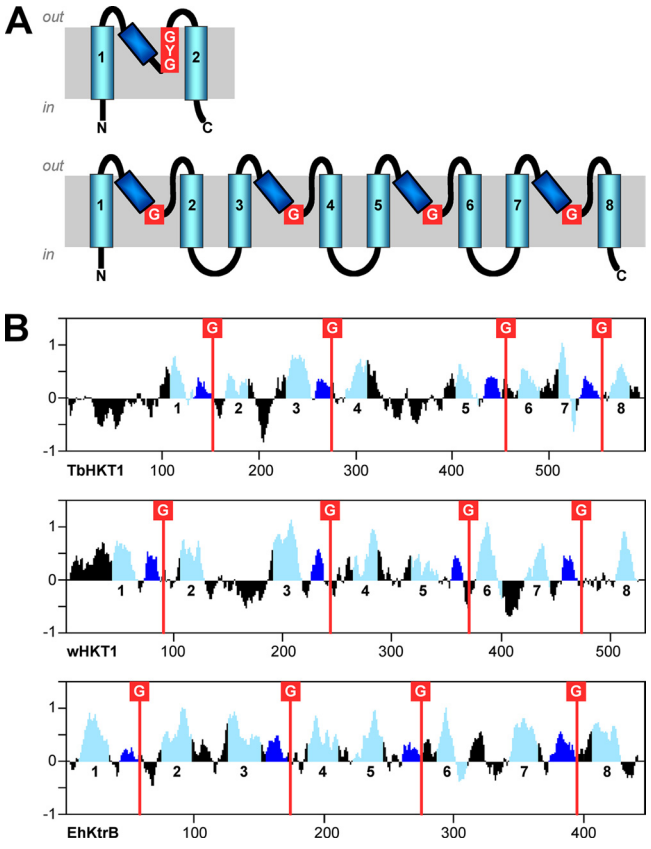

FIG. 2. TbHKT1 carries the hallmarks of Trk/HKT transporters. (A) Schematic representation of the membrane topology of KcsA-type K^+ channels (top) and Trk/HKT transporters (bottom). Transmembrane domains are depicted in light blue, P-loops in dark blue, and K^+ selectivity filters in red. (B) Kyte-Doolittle hydrophobicity, colored according to the same scheme, of TbHKT1, wheat HKT1 (TaHKT2;1), and *Enterococcus hirae* KtrB.

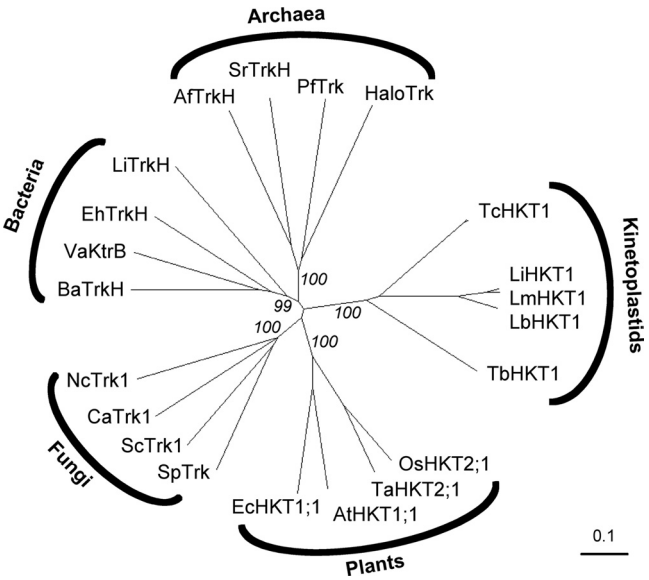

FIG. 3. Phylogenetic tree of the Trk/HKT superfamily with representative members. Bootstrap numbers of the main branches are shown in percent positives of 1,000 rounds. Protein abbreviations and GenBank accession numbers are as follows: *T. cruzi* TcHKT1 (71424637), *L. braziliensis* LbHKT1 (154344371), *L. infantum* LfHKT1 (146100295), *L. major* LmHKT1 (72546739), *Pyrococcus furiosus* PfTrk (18894043), *Archaeoglobus fulgidus* AfTrkK (11498445), *Salinibacter ruber* SrTrkH (83816848), *Halobacterium* HaloTrk (15791310), *Arabidopsis thaliana* AtHKT1;1 (7716474), *Oryza sativa* OsHKT2;1 (14588581), *Triticum aestivum* TaHKT2;1 (567062), *Eucalyptus camaldulensis* EcHKT1;1 (9719299), *Leptospira interrogans* TrkH (24193699), *Vibrio alginolyticus* KtrB (3395637), *Enterococcus hirae* TrkH (G53610), *Bacillus anthracis* TrkH (229604742), *Candida albicans* CaTrk1 (8099700), *Neurospora crassa* NcTrk1 (3724137), *S. cerevisiae* Trk1 (72015), and *Schizosaccharomyces pombe* Trk1 (1182049).

TrkE/SapD (23). However, HMMer searches with TrkA- and TrkE/SapD-specific profiles did not provide any evidence for such accessory proteins in *T. brucei* (not shown).

Functional characterization of TbHKT1 in heterologous expression systems. *TbHKT1* was functionally expressed in *E. coli*, *S. cerevisiae*, and in *Xenopus laevis* oocytes. For expression in *E. coli*, we used the potassium transport-defective quadruple mutant L2003, which lacks the Trk/HKT superfamily genes *TrkG* and *TrkH*, the Kup gene *TrkD*, and the ATPase *Kdp* (46). LB2003 cells need ≥20 mM K^+ in the medium to grow. When *TbHKT1* was expressed in *E. coli* LB2003, the cells grew at K^+ concentrations as low as 2 mM (Fig. 4A). Similar results were obtained when *TbHKT1* was expressed, under the control of a Gal1 promoter, in *S. cerevisiae* strain CY162, a K^+ uptake-defective double mutant that lacks the Trk/HKT genes *Trk1* and *Trk2* (20) and is unable to grow on potassium concentration below 20 mM. Expression of *TbHKT1* allowed the CY162 mutant to grow on 1 mM KCl while control transformants with the empty vector required at least 20 mM KCl for growth (Fig. 4B). As some of the plant HKT proteins function as K^+:Na^+ symporters (42), TbHKT1-mediated K^+ transport was tested for Na^+ dependency. Yeast CY162 mutants cells were still able to grow on 1 mM KCl in the absence of Na^+ (Fig. 4B), indicating that TbHKT1 is not a K^+:Na^+ symporter. To better address the substrate specificity of TbHKT1, the gene was

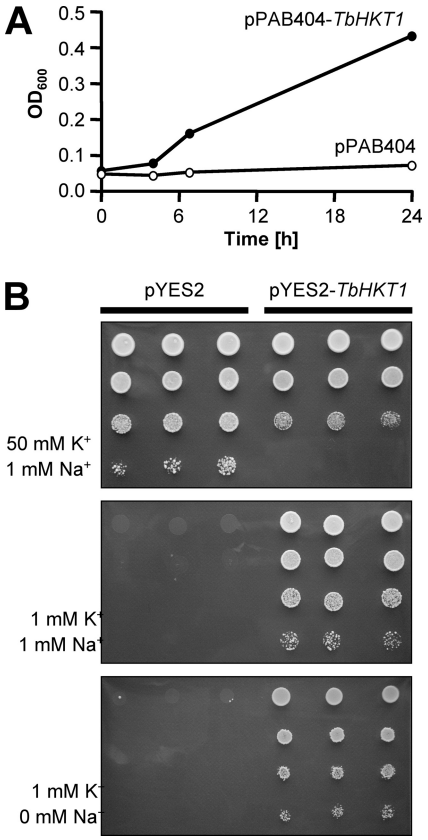

FIG. 4. TbHKT1 suppresses K^+ uptake deficiency in yeast and in *E. coli*. (A) The plasmid containing *TbHKT1* allows the *E. coli* K^+ uptake-deficient quadruple mutant LB2003 to grow in medium containing 2 mM K^+; the empty plasmid does not. (B) Expression of *TbHKT1* in the *S. cerevisiae* Δ*trk1* Δ*trk2* double null mutant CY162 permits growth on 1 mM K^+. The effect is not dependent on Na^+. Cells were spotted in triplicates, in a dilution series from optical density at 600 nm (OD_{600}) of 1 to OD_{600} of 10^{-3}.

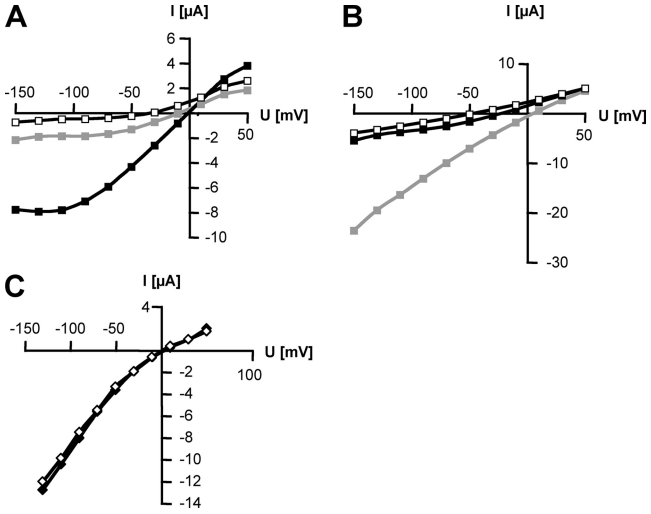

FIG. 5. TbHKT1 mediates K^+ transport in *Xenopus* oocytes. Currents recorded as a function of applied voltage with oocytes expressing *TbHKT1* (A and C) or *AtHKT1* (B), in the presence of 100 mM K^+ (black squares), 100 mM Na^+ (gray squares), or 100 mM Tris (white squares) in the bath solution. Control oocytes injected with water did not exhibit any currents (data not shown). (C) In the presence of 120 mM K^+ in the bath solution, the absence (white diamonds) or presence (black diamonds) of 1 mM Na^+ did not affect the currents.

expressed in *Xenopus* oocytes. A voltage ramp from −150 to 50 mV produced currents only in the presence of external K^+; when K^+ was substituted with Na^+, only small currents were recorded (Fig. 5A). *Arabidopsis* AtHKT1 (51) was used as a positive control for Na^+ transport (Fig. 5B). At 120 mM external K^+, the currents mediated by TbHKT1 were independent of Na^+ (Fig. 5C). Thus, based on the heterologous expression in bacteria, yeast, and oocytes, TbHKT1 is a Na^+-independent K^+ transporter. It is interesting to note that in the only multicellular eukaryotes known to possess *Trk*/*HKT* genes, the vascular plants, the majority of the transporters function in Na^+ homeostasis (39). Mediating root-to-shoot transport of Na^+, they are important for salt tolerance and—with a few exceptions (42)—affect K^+ homeostasis only indirectly (21). The function of TbHKT1 in trypanosomes remains to be elucidated.

Functional characterization of TbHKT1 in trypanosomes. *TbHKT1* is expressed in both procyclic and bloodstream-form *T. b. brucei* trypanosomes (Fig. 6A), albeit at low levels as the Northern blot signals were only obtained with ^{32}P-radiolabeled probes, not with digoxigenin-labeled probes (not shown). Furthermore, *in situ* tagging (38) of TbHKT1 with a hemagglutinin tag did not yield an immunofluorescence signal above background, neither in bloodstream-form nor in procyclic trypanosomes (not shown). To investigate the function of TbHKT1 in trypanosomes, the expression of *TbHKT1* was downregulated

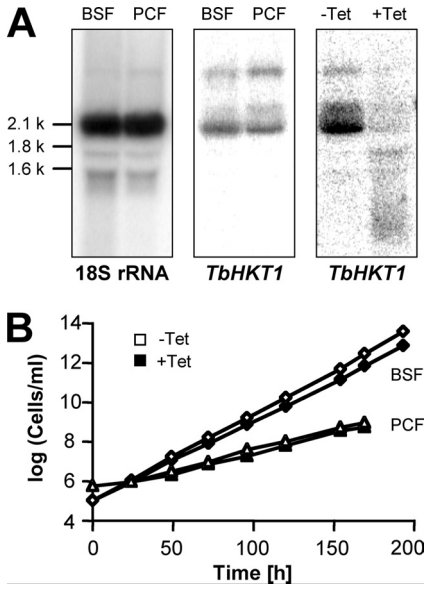

FIG. 6. Expression and silencing of *TbHKT1* in *T. brucei.* (A) Northern blot with total RNA isolated from cultured *T. b. brucei* bloodstream forms (BSF) and procyclic forms (PCF). A probe complementary to the 18S rRNA was used as a loading control (left panel). *T. b. brucei* RNAi cells that express double-stranded *TbHKT1* stem-loop constructs under the control of the Tet operator downregulate *TbHKT1* expression in the presence of tetracycline (right panel). (B) Growth curves with bloodstream-form (diamonds) and procyclic (triangles) *T. b. brucei TbHKT1*-RNAi cells; addition of tetracycline (1 mg/ml) to the medium does not impair growth.

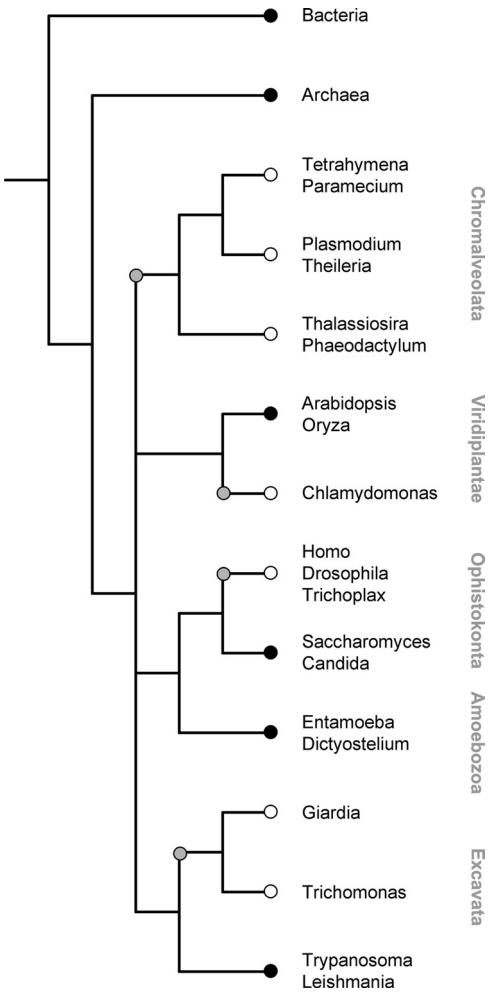

FIG. 7. Repeated loss of *Trk*/*HKT* genes in the major eukaryote lineages. Schematic phylogeny of fully sequenced eukaryotes that possess (black circles) or lack (white circles) genes of the *Trk*/*HKT* superfamily. The gray circles indicate presumed loss of *Trk*/*HKT* genes.

by RNAi-mediated gene silencing. Bloodstream-form *T. b. brucei* NYSM and procyclic-form *T. b. brucei* 29-13 were stably transformed with a stem-loop construct of a *TbHKT1* fragment under the control of the Tet operator. Forty-eight hours after addition of tetracycline (1 mg/ml) to the medium, the *TbHKT1* mRNA steady-state levels were reduced substantially in procyclic (Fig. 6A) as well as in bloodstream-form (not shown) trypanosomes. However, the *TbHKT1* knockdown trypanosomes did not show any growth phenotype when cultivated in standard medium plus tetracycline over 10 days (Fig. 6B), indicating that expression of *TbHKT1* is not required under these conditions or that residual *TbHKT1* expression after RNAi was sufficient to maintain viability. When the K^+ concentration in the medium was lowered from 5 mM to 0.5 mM, the trypanosomes (bloodstream forms) proliferated more slowly; but again, there was no significant difference in growth rate between *TbHKT1*-RNAi cells incubated with (population-doubling time of 27.3 h) or without (population-doubling time of 24.6 h) tetracycline. We were not able, after 10 independent attempts to disrupt the genes with antibiotic resistance markers, to create homozygous *tbhkt1*$^{-/-}$ knockout trypanosomes. Thus, the question of whether, and under which conditions, TbHKT1 is essential for *T. brucei* remains unresolved.

Repeated loss of *Trk*/*HKT* genes from eukaryotes. A number of genomes from endoparasitic protozoa have been fully sequenced. Besides the trypanosomatids (Table 2), we screened the predicted proteomes of *Plasmodium falciparum*, *Theileria parva*, *Entamoeba histolytica*, *Giardia duodenalis*, and *Trichomonas vaginalis* for the presence of Trk/HKT proteins with the same profile that was used to identify TbHKT1. Additional representatives of the major eukaryote groups were included as well: the ciliates *Tetrahymena thermophyla* and *Paramecium tetraurelia*, the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, the amoeba *Dictyostelium discoideum*, and *Trichoplax adhaerens* as an additional metazoan. No complete genomes are available yet from the rhizaria (foraminifers, radiolarians, etc.). When the presence of *Trk*/*HKT* genes is overlaid on the phylogeny of the analyzed organisms, the emerging picture is enigmatic at first sight since the distribution of *Trk*/*HKT* genes among eukaryotes is not coherent (Fig. 7). While the genes occur in kinetoplastids, amoebae, fungi, and vascular plants, they are absent from *Chlamydomonas* (53), *Giardia*, and *Trichomonas*, as well as from all available chromalveolate and metazoan genomes. However, the broad occurrence of *Trk*/*HKT* genes in bacteria and archaea indicates that the primordial eukaryotes possessed Trk/HKT-type K^+ transporters. Thus, the parsimonial interpretation of Fig. 7 is multiple independent gene loss, at least four times among the major eukaryote lineages: within the chromalveolates, the green plants, the ophistokonts, and the excavates (gray circles in Fig. 7). Apparently, HKT/Trk transporters tend to become redundant in the course of evolution of the mechanisms for K^+ uptake and homeostasis. The fact that the trypanosomatids, in contrast to all other excavates analyzed, have retained an *HKT* orthologue suggests that these K^+ transporters fulfill an indispensable function at some point in the life cycle of a trypanosomatid parasite.

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