Molecular Characterization of *Trypanosoma brucei* P-type H\(^{+}\)-ATPases*

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Shuhong Luo¹, Jianmin Fang, and Roberto Docampo²

From the Center for Tropical and Emerging Global Diseases and the Department of Cellular Biology, University of Georgia, Athens, Georgia 30602

Previous studies in *Trypanosoma brucei* have shown that intracellular pH homeostasis is affected by inhibitors of H\(^{+}\)-ATPases, suggesting a major role for these pumps in this process (VanderHeyden, N., Wong, J., and Docampo, R., (2000) Biochem. J. 346, 53–62). Here, we report the cloning and sequencing of three genes (*TbHA1, TbHA2*, and *TbHA3*) present in the genome of *T. brucei* that encode proteins with homology to fungal and plant P-type proton-pumping ATPases. Northern and Western blot analyses revealed that these genes are up-regulated in procyclic trypanomastigotes. *TbHA1, TbHA2*, and *TbHA3* complemented a *Saccharomyces cerevisiae* strain deficient in P-type H\(^{+}\)-ATPase activity, providing genetic evidence for their function. Indirect immunofluorescence analysis showed that TbHA proteins are localized mainly in the plasma membrane of procyclic forms and in the plasma membrane and flagellum of bloodstream forms. *T. brucei* H\(^{+}\)-ATPase genes were functionally characterized using double-stranded RNA interference methodology. The induction of double-stranded RNA (RNA interference) caused growth inhibition, which was more accentuated in procyclic forms and when expression of all TbHA proteins was decreased. Knockdown of *TbHA1* and *TbHA3*, but not of *TbHA2*, resulted in cells with a lower steady-state pH\(_i\) and a slower rate of pH\(_i\) recovery from acidification. No evidence was found of an intracellular P-type H\(^{+}\)-ATPase activity. These results establish that *T. brucei* H\(^{+}\)-ATPases are plasma membrane enzymes essential for parasite viability.

Sleeping sickness, also known as human African trypanosomiasis, is caused by protozoan parasites within the *Trypanosoma brucei* complex. These parasites have a digenetic life cycle with two main stages: the bloodstream form, which lives in the bloodstream of its mammalian host, and the procyclic form, which lives in the insect vector. The procyclic form replicates within the alimentary tract of the tsetse fly, where the environmental (extracellular) pH is acidic (1), whereas the bloodstream form propagates in the host’s blood, where the extracellular pH is consistently 7.4. Marked differences in metabolism and in intracellular pH (pH\(_i\)) regulation between the two stages have evolved as a result of the very different environments in which they reside (2).

The ability of *T. brucei* to regulate its pH\(_i\) throughout its life cycle is critical to its survival, as most intracellular enzymes function optimally within a narrow pH range. In most higher eukaryotic cells whose environment is neutral, metabolically generated protons are exchanged for Na\(^+\) via an Na\(^+\)/H\(^+\) antiporter (3). The sodium gradient generated by the Na\(^+\)/K\(^+\)-ATPase drives this exchange. However, at low extracellular pH, Na\(^+\)/H\(^+\) exchange is unfavorable, and cells that maintain a pH\(_i\) that is more alkaline than their environment therefore frequently utilize ATP-driven proton pumps to regulate their pH\(_i\). Proton pumping is electrogenic and is usually supported by one or more anion or cation conductive pathways that serve to partially dissipate the membrane potential, thereby increasing the efficiency of the pump. In addition, the protonmotive force generated by these pumps frequently drives nutrient uptake in these cells (2).

On the basis of studies performed with inhibitors of proton pumps, it has been suggested that, although a plasma membrane H\(^{+}\)-ATPase is a major regulator of pH\(_i\) (2) and membrane potential (ΔΨ) (4) in procyclic forms, it plays a minor role in bloodstream forms, where pyruvate efflux is more important for pH\(_i\) regulation, and an ouabain-sensitive sodium pump is more relevant for ΔΨ regulation. In this regard, bloodstream forms live in the neutral pH, constant ion environment of the mammalian bloodstream, where the need to maintain ΔΨ could be satisfied by an Na\(^+\)/K\(^+\)-ATPase that causes K\(^+\) uptake and Na\(^+\) efflux. Because the ion concentrations in blood are held constant, the plasma membrane of bloodstream forms of *T. brucei*, like that of most mammalian cells, can be relatively permeable to cations (2). Additionally, in a neutral pH environment, a passive rather than an active mechanism of regulation of pH\(_i\) is likely to dominate, lessening the need for a plasma membrane H\(^{+}\)-ATPase (2, 4). Similarly, a Na\(^+\)/K\(^+\)-ATPase appears to play an important role in regulating the membrane potential of *Trypanosoma cruzi* bloodstream forms, which are also more permeable to cations than are the other life stage forms (5).

As with yeast (6), the lack of inhibitors specific enough for *in vivo* experiments makes it necessary to perform molecular studies of the *T. brucei* plasma membrane H\(^{+}\)-ATPases to elucidate their importance. H\(^{+}\)-ATPases within the P-type ATPase family are proton pumps driven by the hydrolysis of
Molecular Characterization of T. brucei P-type H\textsuperscript{+}-ATPases

ATP. These pumps were found initially only in the plasma membrane of plants and fungi (7). A sequence analysis of conserved core sequences of all P-type ATPases has grouped them into five subfamilies designated types I–V (8). Type III covers H\textsuperscript{+}-ATPases (type IIIA) and a small group of Mg\textsuperscript{2+}-ATPases from bacteria (type IIIIB). All fungal P-type H\textsuperscript{+}-ATPases compose one subcluster within type IIIA; the plant enzymes compose a second subcluster; and sequences found in Leishmania donovani made up a third subcluster (8). In previous work, we reported that the sequences of T. cruzi P-type H\textsuperscript{+}-ATPase-1 and -2 (TcHA1 and TcHA2, respectively) belong to the same subcluster as the L. donovani sequences and provided genetic and biochemical evidence of the role of their protein products in H\textsuperscript{+} transport (9). The presence of several genes encoding H\textsuperscript{+}-ATPases is frequent in plants as well as in microorganisms, where several isoforms with distinct catalytic or regulatory properties may coexist in the same cell (10, 11). For example, the PMA2 gene product in yeast shows 89% identity to the PMA1 gene product (12), although PMA2 is expressed at very low levels and is not essential for growth (13).

The absence of P-type H\textsuperscript{+}-ATPases in mammalian cells (8, 10) and their presence in fungi has led to the proposal that these pumps are promising targets for antifungal therapy (14), and a similar proposal could be made regarding trypanosomatids (15). In this regard, only electroneutral P-type H\textsuperscript{+}/K\textsuperscript{+}-ATPases belonging to the same family as the Na\textsuperscript{+}/K\textsuperscript{+}-ATPases are present in the plasma membrane of some mammalian cells (16). In the present study, we report the cloning and sequencing of three T. brucei genes that encode proteins with homology to the T. cruzi and L. donovani P-type H\textsuperscript{+}-ATPases. T. brucei H\textsuperscript{+}-ATPases are more abundant in procyclic than in bloodstream stages. The function of these ATPases was studied using RNA interference (RNAi)\textsuperscript{3} and a yeast strain deficient in P-type H\textsuperscript{+}-ATPase (RS-72). The subcellular localization of the H\textsuperscript{+}-ATPases in T. brucei was also investigated.

EXPERIMENTAL PROCEDURES

Culture Methods for T. brucei—The procyclic form 29-13 cell line and the bloodstream form BF cell line (114hgy5-328, also known as the “single-marker” cell line), coexpressing T7 RNA polymerase and the tetracycline repressor were gifts from Dr. George A. M. Cross (Rockefeller University) (17). Procyclic forms were diluted to 1 × 10\textsuperscript{6} cell/ml and bloodstream forms to 0.5 × 10\textsuperscript{5} cell/ml and cultured in the appropriate media. Growth curves were plotted using the product of the cell density and the dilution factor.

Yeast Strains and Culture Conditions—Saccharomyces cerevisiae strain RS-72 (MATa ade1-100 his4-519 leu2-3,112) (10), carrying the yeast PMA1 gene under the control of the galactokinase gene (GAL1) promoter, was used for transformation with LEU2 plasmids (20). Yeast cells were grown in SGAHL medium (synthetic medium containing 2% (w/v) galactose, 0.7% (w/v) yeast nitrogen base without amino acids (Difco), 0.2 mM adenine, 0.4 mM histidine, and 1 mM leucine). Yeast cells were made competent for plasmid uptake by treatment with lithium acetate and polyethylene glycol according to Gietz et al. (21). Positive transformants were selected in SGAH medium (SGAHL medium without leucine) after 4 days of growth at 30 °C. The new strains (bearing the respective plasmids) were named MP625 (pMP625), RS1002 (pRS890), RD0511 (pRD051), RD0522 (pRD052), and RD0533 (pRD053). Transformants were maintained in SGAH medium or transferred to medium containing 2% (w/v) glucose (dextrose) in place of galactose (SDAH medium). The media were buffered with 50 mM succinic acid adjusted to pH 5.5. Solid media contained 2% agar (Difco).

Chemicals—Fetal and newborn calf serum, Dulbecco’s phosphate-buffered saline (DPBS), Tween 20, anti-α-tubulin antibody, and poly-l-lysine-treated slides were purchased from Sigma. Monoclonal antibody CLP001A against T. brucei procyclin was purchased from Cedarlane Laboratories Ltd. (Ontario, Canada). Fluorescein-labeled antibodies and 2’,7’-bis(2-carboxyethyl)-(5(6)-carboxyfluorescein were from Molecular Probes (Eugene, OR). TRIZol reagent, SuperScript PCR buffer, SuperScript II reverse transcriptase, the DNA ladder, the PCR2.1 TA cloning kit, and Taq polymerase were from Invitrogen. Restriction enzymes and the PolyATtract\textsuperscript{R} mRNA isolation system were from Promega Corp. (Madison, WI). The Marathon cDNA amplification kit was from Clontech (Mountain View, CA). Sequenase was from U. S. Biochemical Corp. (Cleveland, OH). [α\textsuperscript{32P}]dCTP (3000 Ci/mmol) was from GE Healthcare. Zeta-Probe GT nylon membranes, prestoned molecular mass standards, and the protein assay were from Bio-Rad. Pfu DNA polymerase was from Stratagene (La Jolla, CA). All other reagents were analytical grade. Plasmid pMP625, derived from YEp351 (20) and containing the promoter and terminator of PMA1, and plasmid pRS890 (22), containing the yeast PMA1 gene, were kindly provided by Dr. Michael G. Palmgren (University of Copenhagen, Copenhagen, Denmark).

Cloning of Plasma Membrane-type Proton ATPase (TbHA)—A TBLASTN search of all available sequence data bases using the amino acid sequence of the P-type H\textsuperscript{+}-ATPase of T. cruzi (GenBank\textsuperscript{TM} accession number AF254412 (9) identified three contigs in the T. brucei genome data base in chromosome 10 (TRY10.0.001893, 52,000 bp; contig 12146, 52,000 bp; TRY10.0.001690, 4070 bp; available at www.sanger.ac.uk/Projects/T_brucei/) and a partial sequence called the proton-motive P-type ATPase TBH1 (GenBank\textsuperscript{TM} accession number AF145721; 773 bp) in the NCBI Database (available at www.ncbi.nlm.nih.gov/ntrevoir/viewer.fcgi?val(AF145721.1). Sequence analysis showed that the three contigs encode three putative P-type ATPases designated TbHA1, TbHA2, and TbHA3. Based on this information, a 1.2-kb cDNA fragment, which is identical in

\textsuperscript{3} The abbreviations used are: RNAi, RNA interference; DPBS, Dulbecco’s phosphate-buffered saline; contigs, groups of overlapping clones; RACE, rapid amplification of cDNA ends; Mops, 4-morpholinopropanesulfonic acid.
Molecular Characterization of T. brucei P-type H\(^+\)-ATPases

the central regions of \(TbHA1\), \(TbHA2\), and \(TbHA3\), was cloned and sequenced using the reverse transcription-PCR technique with gene-specific primers \(TbP51\) (5'-TCACGATCTCGGT-TCACTG-G-3') and \(TbP31\) (5'-TTGCACAAACAAAGATTT-GG-3'). To obtain the full-length cDNA, the rapid amplification of cDNA ends (RACE) technique (23) was performed using a Marathon cDNA amplification kit and primer \(Tb-5'-SL\) (5'-AAC-GCTATTATAGAAGATTTCTG-3') and specific primers \(TbP31\), \(TbP32\) (5'-CTGCAAGGATCAGGGA-3'), and \(TbP33\) (5'-TGAATACGTCTCGATGTTCT-3') for \(5'-\)RACE and an oligo(dT) primer (5'-ATGAGTATCGTCGCAGTTCTC-3') for \(3'-\)RACE and specific primers \(TbP51\), \(TbP52\) (5'-GCTAAGTCGTCTAAGGAAACC-3'), and \(TbP53\) (5'-TGCATGCTAATGGAGG-3') for \(3'-\)RACE. PCR products were cloned into the pCR2.1TA vector and sequenced using an ABI PRISM\textsuperscript{®} BigDye\textsuperscript{™} Terminator Version 3.0 Cycle Sequencing Ready Reaction kit.

Sequence Analysis—DNA sequence data were generated at the High Throughput Sequencing and Genotyping Unit of the Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. Sequence analysis was done using Biology WorkBench Version 3.2 (available at workbench.sdsc.edu) and Wisconsin Package Version 10.0-UNIX (Genetics Computer Group, Inc., Madison, WI). The PSORT program was from Kenta Nakai (University of Tokyo, Tokyo, Japan). The complete gene sequences for \(TbHA1\), \(TbHA2\), and \(TbHA3\) were submitted to the GenBank\textsuperscript{TM} Data Bank with accession numbers AF507072, AY115557, and AY263211, respectively.

Southern and Northern Blot Analyses—DNA was isolated by standard procedures (24). Total RNA was isolated with TRIzol reagent following the manufacturer’s recommendations. The polyadenylated RNA was obtained using the PolyAtract\textsuperscript{®} mRNA isolation system. DNA was run on 1.0% agarose gels with 2.2M formaldehyde, and transferred to Zeta-Probe GT nylon membranes. RNA was electrophoresed on 1.0% agarose gels with 2.2 M formaldehyde, 20 mM Mops (pH 7.0), 8 mM sodium acetate, and 1 mM EDTA and transferred to Zeta-Probe GT nylon membranes. DNA probes were prepared using random hexanucleotide primers, Klenow fragment of DNA polymerase I (Prime-a-Gene system), and [\(\alpha-\)\textsuperscript{32}P]dCTP. The hybridized filters were washed under high stringency conditions (0.1% standard saline citrate and 0.1% SDS at 65 °C) unless indicated otherwise.

The \(\alpha\)-tubulin (\(TUB1\)) fragment, used as a control in Northern blot analyses, was obtained from pZJM (25), kindly provided by Dr. Paul T. England (Johns Hopkins University). Comparison of the levels of \(TbHA1\) and \(TbHA2\) transcripts in procyclic and bloodstream forms was done, taking as a reference the densitometric values obtained with the \(TUB1\) transcripts and assuming a similar level of expression of this gene in both stages.

**SDS Electrophoresis and Preparation of Western Blots—** Samples of total lysates from \(T. brucei\) procyclic and bloodstream stages (20 \(\mu\)g of protein in 10 \(\mu\)l) were mixed with 10 \(\mu\)l of 125 mM Tris-Cl (pH 7), 10% (w/v) mercaptoethanol, 20% (w/v) glycerol, 4.0% (w/v) SDS, and 4.0% (w/v) bromphenol blue as tracking dye and boiled for 5 min before application to 10% SDS-polyacrylamide gels. Electrophoresed proteins were transferred to nitrocellulose with a Bio-Rad Trans-Blot apparatus. After transfer, the nitrocellulose was blocked overnight in 5% nonfat dry milk in 0.1% Tween 20/DPBS (Twen/DPBS) at 4 °C. A 1:10,000 dilution of polyclonal rabbit serum against TcHAf in Twen/DPBS was then applied at room temperature for 60 min. The nitrocellulose was washed three times for 15 min each with Twen/DPBS and incubated with secondary antibody (1:20,000) at room temperature for 60 min. Immunoblots were visualized on radiographic film using the ECL enhanced chemiluminescence detection kit (Amersham Biosciences) according to the instructions of the manufacturer.

**Immunofluorescence Microscopy—** Parasites fixed with 4% formaldehyde were allowed to adhere to poly-1-lysine-coated coverslips, permeabilized with 0.3% Triton X-100 for 5 min, blocked with 3% bovine serum albumin in DPBS, and incubated with a 1:100 dilution of anti-TcHAf antibody (9) and a 1:160 dilution of fluorescein isothiocyanate-coupled goat anti-rabbit IgG secondary antibody. Control preparations were incubated with preimmune serum (1:100) or without the primary antibody. After being washed, slides containing the treated trypanosomes were mounted with anti-fade VECTASHIELD medium (Vector Laboratories). Immunofluorescence images were obtained with an Olympus BX-60 fluorescence microscope. The images were collected with a system consisting of a CCD camera (Model CH 250), an electronic unit (Model CE 200A, equipped with a 50-Hz 16-bit A/D converter), and a controller board (Model NU 200) (all from Photometrics Ltd., Tucson, AZ). Images were acquired and evaluated using Adobe Photoshop on a Macintosh Quadra 840AV computer.

**Deconvolution Fluorescence Microscopy—** Fluorescence microscopy and digital image collection were performed on an Olympus IX-71 inverted fluorescence microscope with a Photometrics Coolsnap\textsuperscript{®}, CCD camera driven by DeltaVision software (Applied Precision, Seattle, WA). Differential interference contrast and fluorescent optical images were taken through cells, and DeltaVision software (softWoRx) was used to deconvolve these images.

**Double-stranded RNA Expression and Trypanosome Transfection—** DNA fragments corresponding to the N-terminal flanking and coding regions of \(TbHA1\) and \(TbHA3\) from nucleotides 43 to 294 and \(TbHA2\) from nucleotides 62 to 349 were amplified by reverse transcription-PCR from \(T. brucei\) procyclic stage cDNA using primers P15 (5'-CTCGAGAGCTCAGGTAGTAAAG-GGC-3') and P13 (5'-CTAGAGAAGCTGTTACGCAC-TATG-3') and primers P25 (5'-CTCGAGAGGCACTTATAAG-GACACAG-3') and P23 (5'-CTAGATGATGCTGTCGAGCATCTC-3') with the XhoI and XbaI linkers underlined, respectively. To inhibit the expression of \(TbHA1\), \(TbHA2\), and \(TbHA3\) simultaneously, DNA fragments corresponding to the central coding regions of \(TbHA1\), \(TbHA2\), and \(TbHA3\), which are identical, from nucleotides 1176 to 1880 of \(TbHA1\) and \(TbRNAiP3\) (5'-GCTCGAGATTCCACCCCTTACAGTG-3') and \(TbRNAiP3\) (5'-CTGA-GATGATGCTGTCGAGCATCTC-3') with the XhoI and XbaI linkers underlined). The PCR fragments were cloned into the pCR2.1TA vector and sequenced using the ABI PRISM\textsuperscript{®} BigDye\textsuperscript{™} Terminator Version 3.0 Cycle Sequencing Ready Reaction
Molecular Characterization of T. brucei P-type H\(^{+}\)-ATPases

**RESULTS**

Cloning and Sequence Analysis of T. brucei H\(^{+}\)-ATPases—To screen for genes encoding P-type H\(^{+}\)-ATPases in T. brucei, the

RNAi was induced by addition of 1 \(\mu\)g/ml tetracycline to the medium.

Expression of TbHA1, TbHA2, and TbHA3 in Yeast—The full-length coding regions of TbHA1, TbHA2, and TbHA3 were amplified from T. brucei procyclic form genomic DNA using primers YA1P51 (5'-CTCGAGAAGATGGGGGATCTGG3', with the XhoI site underlined) and YA1P31 (5'-ACTAGTTTAATAGGTTCCTTAGAC3', with the Spel site underlined), primers YA2P51 (5'-CTCGAGAAATGGCATATGGTGGC3') and YA2P31 (5'-ACTAGTTTAATAGGTTCCTTAG3'), and primers YA3P51 (5'-CTCGAGAAGATGGGGAATCTGG3') and YA3P31 (5'-ACTAGTTTAATAGGTTCCTTAG3'), respectively. The

FIGURE 1. **Southern, Northern, and Western blot analyses of TbHA.** A, Southern blot analysis of the TbHA genes in genomic DNA from T. brucei procyclic trypomastigotes. Total genomic DNA (10 \(\mu\)g/lane) was digested with restriction enzymes BamHI (lane 1), EcoRI (lane 2), Scal (lane 3), XbaI (lane 4), and XhoI (lane 5); electrophoresed; blotted, and probed at high stringency with probe a (right panel). Equal amounts of mRNA were observed under UV light in each lane (left panel). The membrane was stripped and probed with a \(^{32}\)P-labeled 0.65-kb fragment of the \(\alpha\)-tubulin (\(\alpha\)-Tub) gene from T. brucei as a loading control (center panel). B, ethidium bromide. C, Western blot analysis of TbHA. 20 \(\mu\)g of protein from bloodstream and procyclic forms was subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with rabbit anti-TcHA1 antibody (upper panel) and probed with anti-\(\alpha\)-tubulin monoclonal antibody (lower panel). The arrows indicate the 85- and 100-kDa TbHA bands.

For stable transfection of the procyclic form host strain 29-13, the protocol was performed as described by LaCount et al. (26). Briefly, procyclic forms (1 \(\times\) 10⁷ cells/ml) were washed once with 5 ml of cytomix buffer (27) and resuspended in 0.5 ml of cytomix buffer containing 10 \(\mu\)g of plasmid that had been linearized by NotI digestion so that it could target the rDNA spacer region (28). Transfections were carried out in 4-mm cuvettes using a BTX electroporator with peak discharge at 1.5 kV and 25 microfarads. Immediately following transfection, parasites were transferred into 10 ml of SDM-79 supplemented with G418 and hygromycin. After 24 h, selection was applied by addition of G418, and 1-ml aliquots were plated onto 24-well tissue culture plates. After 24 h, selection was applied by addition of 2.5 \(\mu\)g/ml phleomycin, and the medium was changed everyday for 3 days. Stable clones were evident after 7–10 days.

kit and ligated into the XhoI and XbaI sites of the RNAi vector p2T7 (26).

For stable transfection of the procyclic form host strain 29-13, the protocol was performed as described by Sim et al. (29). Briefly, for each transfection, bloodstream forms of the parasites were grown to mid-log phase (5 \(\times\) 10⁶ cells/ml), and 2.5 \(\times\) 10⁷ cells were collected and washed once with 5 ml of cytomix buffer (27) and resuspended in 0.5 ml of cytomix buffer containing 10 \(\mu\)g of plasmid that had been linearized with NotI digestion so that it could target the rDNA spacer region (28). Transfections were carried out in 4-mm cuvettes using a BTX electroporator with peak discharge at 1.5 kV and 25 microfarads. Immediately following transfection, parasites were transferred into 10 ml of SDM-79 supplemented with G418 and hygromycin. After 24 h, selection was applied by culturing in the presence of 2.5 \(\mu\)g/ml phleomycin, and the parasites were grown for 2 or 3 weeks to form stable lines. Clonal lines were obtained by limiting dilution. For induction of double-stranded RNA, parasites were cultured in the same medium containing 1.0 \(\mu\)g/ml tetracycline. Parasites were diluted 10-fold when densities reached a minimum of 1 \(\times\) 10⁶ cells/ml. They were not allowed to grow beyond 5 \(\times\) 10⁶ cells/ml before diluting again.

In the case of the bloodstream host strain BF cell line, the protocol was as described by Morris et al. (29). Briefly, for each transfection, bloodstream forms of the parasites were grown to mid-log phase (5 \(\times\) 10⁶ cells/ml), and 2.5 \(\times\) 10⁷ cells were collected and washed once with 5 ml of cytomix buffer (27) and resuspended in 0.5 ml of cytomix buffer containing 10 \(\mu\)g of plasmid that had been linearized with NotI digestion so that it could target the rDNA spacer region (28). Transfections were carried out in 4-mm cuvettes using a BTX electroporator with peak discharge at 1.5 kV and 25 microfarads. Immediately following transfection, parasites were transferred into 24 ml of HMI-9 supplemented with G418, and 1-ml aliquots were plated onto 24-well tissue culture plates. After 24 h, selection was applied by addition of 2.5 \(\mu\)g/ml phleomycin, and the medium was changed everyday for 3 days. Stable clones were evident after 7–10 days.
amino acid sequences of P-type H\textsuperscript{+}-ATPases from T. cruzi (9) were used to search all available sequence data bases using TBLASTN. This search yielded three putative open reading frames designated TbHA1, TbHA2, and TbHA3, which have a high identity to T. cruzi P-type H\textsuperscript{+}-ATPases (76%). On the basis of this information, we cloned TbHA1, TbHA2, and TbHA3 by reverse transcription-PCR. A 1.2-kb cDNA fragment was used to design appropriate gene-specific primers for the generation of 5'- and 3'-end DNA fragments using the RACE method (23) and for the reconstruction of full-length cDNAs. The nucleotide sequences of 3637 bp (TbHA1), 3728 bp (TbHA2), and 3631 bp (TbHA3) revealed open reading frames of 2739 bp (nucleotides 78–2816), 2718 bp (nucleotides 219–2936), and 2763 bp (nucleotides 78–2840), which encode proteins of 912, 905, and 920 amino acids with relative molecular masses of 100.2, 99.5, and 100.6 kDa and calculated pI values of 5.86, 7.30, and 5.86, respectively. When the cDNA sequences were compared with the genomic sequences from chromosome 10, the predicted translation initiation sites of TbHA1, TbHA2, and TbHA3 were preceded by 43, 95, and 43 bp of 5'-untranslated sequences. The polyadenylation sites of TbHA1, TbHA2, and TbHA3 were preceded by 780, 776, and 776 bp, respectively.

Molecular Characterization of T. brucei P-type H\textsuperscript{+}-ATPases

![Figure 2. ClustalW alignment of putative H\textsuperscript{+}-ATPase amino acid sequences from T. brucei (TbHA1, GenBank™ accession number AF507072; TbHA2, accession number AY115557; and TbHA3, accession number AY263211), T. cruzi (TcHA2, accession number AF254412), L. donovani (LmHA1, AF109296), and S. cerevisiae (Pma1, Z72530). Identical residues are shaded. Amino acid residues absent from other sequences are denoted by dashes. The amino acid sequences corresponding to the conserved catalytic autophosphorylation and ATP-binding regions are underlined. Transmembrane domains I–X and potential N-glycosylation sites are indicated by dashed lines and asterisks above the sequences, respectively. Boxes indicate motifs specific to type IIA P-type ATPases. Arrowheads show conserved residues that have been studied by site-directed mutagenesis.

![Figure 3. Deconvolution immunofluorescence microscopy showing the localization of TbHA in procyclic and bloodstream forms of T. brucei. Bloodstream forms (BF; C) and procyclic forms (PF; A) were incubated with rabbits anti-TcHAf antibody. B and D show the same cells as in A and C, respectively, by differential interference contrast (DIC) microscopy. Arrows show plasma membrane labeling. Scale bars = 5 μm. DAPI, 4',6-diamidino-2-phenylindole.](image-url)
Southern blot analysis was performed with a 650-bp DNA probe (probe a), which would hybridize to all three TbHA genes at the central regions, to confirm the presence of these genes in the T. brucei genome (Fig. 1A). Most restriction enzymes used produced multiple hybridization bands. Together with the reverse transcription-PCR, 5'H11032-RACE, and 3'H11032-RACE results, these data were consistent with the presence of TbHA as a multiple-copy gene.

Structure of the Coding Region of the TbHA Genes—TbHA genes encode proteins with molecular masses of ~100 kDa. This size is consistent with those reported for other proton pumps in plants and fungi (30). A BLASTP search of protein data bases showed that TbHA1, TbHA2, and TbHA3 are closely related to the putative P-type H⁺-ATPases from other trypanosomatids. TbHA1, TbHA2, and TbHA3 have 80, 82, and 79% identities and 90, 91, and 88% similarities to T. cruzi H⁺-ATPases and 79, 79, and 80% identities and 88, 88, and 89% similarities to L. donovani H⁺-ATPases, respectively. The next highest BLAST matches were identified as putative H⁺-ATPases from plants, fungi, various algae, the slime mold Dictyostelium discoideum, and the apicomplexan parasite Toxoplasma gondii (29–35% identities and 46–53% similarities).

Hydropathy analysis revealed a profile very similar to those of other P-type ATPases, with 10 transmembrane domains, as shown in Fig. 2. The sequences contain two motifs common to all P-type ATPases, which were the basis of the original PCR primers (underlined in Fig. 2). The first of these is DKTGT(LIVM)(TIS) (PROSITE motif PS00154; available at www.expasy.org/prosite), which starts with the aspartate that is phosphorylated during the catalytic reaction. The second of these is GDGND (8), the hinge sequence linking the large cytosolic domain to the membrane-associated C-terminal domain of P-type ATPases (10). The TbHA sequences also contain all of the amino acid residues and short peptides that

![FIGURE 4. Effect of inhibition of TbHA1, TbHA2, and TbHA3 expression on growth of procyclic and bloodstream forms. Procyclic forms (PF; A–C) were grown in the absence (Not induced, •) or presence (Induced, ○) of 1 μg/ml tetracycline. The transfected cell lines were p2T7/TbHA1,2,3 (A), p2T7/TbHA1,3 (B), and p2T7/TbHA2 (C). Bloodstream forms (BF; D–F) were grown in the absence (Not induced, •) or presence (Induced, ○) of 1 μg/ml tetracycline. The transfected cell lines were clone 1B1 containing p2T7/TbHA1,2,3 (D), clone 1A4 containing p2T7/TbHA1,3 (E), and clone 2C1 containing p2T7/TbHA2 (F). RNA of procyclic forms (transfected by p2T7/TbHA1,2,3) grown for 0, 1, 2, and 3 days in the presence of tetracycline (1 μg/ml) was analyzed by Northern blotting (G). Poly(A) RNA (3 μg/lane) was subjected to gel electrophoresis before transfer to a nylon membrane and then hybridized with 32P-labeled probe a. As a control, the blot was also reprobed with α-tubulin (α-tub). T. brucei procyclic form (transfected by p2T7/TbHA1,2,3) lysates (20 μg of protein/lane) from tetracycline-induced cells were analyzed by immunoblotting on days 0, 2, 4, 6, 8, and 10 of culture (H). Rabbit anti-TcHAf antibody and monoclonal antibody CLP001A against procyclin (CLP), a specific glycoprotein antigen found in procyclic forms of T. brucei, were used to reveal TbHA and procyclin, respectively.

Southern blot analysis was performed with a 650-bp DNA probe (probe a), which would hybridize to all three TbHA genes at the central regions, to confirm the presence of these genes in the T. brucei genome (Fig. 1A). Most restriction enzymes used produced multiple hybridization bands. Together with the reverse transcription-PCR, 5’-RACE, and 3’-RACE results, these data were consistent with the presence of TbHA as a multiple-copy gene.
are common to type IIIA P-type ATPases but are not preserved in other subgroups of P-type ATPases (boxed in Fig. 2) (2). A number of amino acids known, from site-directed mutagenesis studies, to have a role in other H\(^{+}\)-ATPases are also conserved in the \(T. brucei\) proteins. Mutation of \(S. cerevisiae\) Gly\(^{125}\), Gly\(^{125}\), and Gly\(^{125}\) in \(TbHA1, TbHA2,\) and \(TbHA3,\) respectively (Fig. 2, first arrowhead) confers a hygromycin resistance phenotype (31). Mutation of Asp\(^{790}\) in \(S. cerevisiae\) (\(T. brucei\) Asp\(^{790},\) Asp\(^{791},\) and Asp\(^{790}\)) (Fig. 2, second arrowhead) abolishes ATPase activity and proton transport (32). The combined presence of these features suggests a close relationship of the \(T. brucei\) enzymes to the fungal and plant group of proton-pumping ATPases. The most striking feature noticed upon comparison of TbHA1, TbHA2, and TbHA3 is the cluster of changes that occurred at the N and C termini of the proteins. More differences are located at the N-terminal 80 and C-terminal 30 amino acids (Fig. 2). We observed a total of 20 amino acid differences in the central regions. One amino acid change is located in hydrophobic domain I, but this change did not alter the hydrophobicity of the domain. Similarly, the single amino acid changes in domains III and X are also conservative.

Expression of \(T. brucei\) P-type ATPase Genes—Northern blot analysis of poly(A) RNA extracted from procyclic and bloodstream forms of the parasite was performed with probe a, which would hybridize to transcripts from either ATPase gene. The analysis revealed the existence of a main transcript of 3.7 kb (Fig. 1B, right panel). The level of \(TbHA\) mRNA was greater in procyclic than in bloodstream forms (32). We observed a total of 20 amino acid differences in the central regions. One amino acid change is located in hydrophobic domain I, but this change did not alter the hydrophobicity of the domain. Similarly, the single amino acid changes in domains III and X are also conservative.

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Molecular Characterization of \(T. brucei\) P-type H\(^{+}\)-ATPases—We investigated the localization of the TbHA proteins in \(T. brucei\) by immunocytochemistry with anti-TcHAf antibody (9). The reaction of this antibody in procyclic and bloodstream forms of \(T. brucei\) as revealed with fluorescein-labeled secondary antibody was of variable intensity. Deconvolution of the images taken allowed us to detect strong labeling at the cell surface of the procyclic forms (Fig. 3A, arrow). Labeling of bloodstream forms (see Fig. 4H) was much weaker (Fig. 3C), in agreement with the lower expression of these pumps in these stages (Fig. 1, B and C). No fluorescence was observed in control parasites incubated only in the presence of fluorescein-labeled goat anti-rabbit IgG secondary antibody or preimmune serum (data not shown).

\(TbHA\) Genes Are Essential in \(T. brucei\)—We investigated the importance of \(TbHA\) genes for growth by RNAi. An inactivating 500-bp cDNA fragment containing the 5′-end untranslated region and the 5′-ends of \(TbHA1\) and \(TbHA3\) or of \(TbHA2\) or a central region common to all \(TbHA\) open reading frames was inserted using the p2T7\(^{11\text{B}}\) vector (26). Procyclic forms (29-13) of \(T. brucei\) that express the tetracycline repressor and the bacterial T7 RNA polymerase were transfected with the RNAi constructs directed against \(TbHA1,3, TbHA2,\) and \(TbHA1,2,3.\) This resulted in cells that survived phleomycin selection. When production of double-stranded RNA was induced by tetracycline, growth of cells deficient in all genes (Fig. 4) was completely inhibited. However, growth was affected in cells deficient in each individual H\(^{+}\)-ATPase, but the knockdown was not lethal (Fig. 4, A–C). Northern (Fig. 4G) and Western (Fig. 4H) blot analyses of \(TbHA\) RNA from procyclic forms grown in
the presence of tetracycline showed reduction of intact TbHA RNA and TbHA protein, respectively.

We investigated the localization of TbHA in mutant procyclic forms grown in the presence or absence of tetracycline by immunocytochemistry with anti-TcHAf antibody (9). The reaction of this antibody in uninduced mutant procyclic forms showed colocalization at the surface membrane with the reaction produced using anti-procyclin antibody (Fig. 5, A–C). In the induced cells, labeling of the surface greatly decreased (Fig. 5, E–G), and some rounded parasites were visible. No fluorescence was observed in control parasites incubated only in the presence of fluorescein-labeled goat anti-rabbit IgG secondary antibody or preimmune serum (data not shown).

Similar RNAi experiments were done with bloodstream forms. When production of double-stranded RNA was induced by tetracycline, growth of cells deficient in all genes (TbHA) was completely inhibited. However, growth was affected in cells deficient in each individual H/H11001-ATPase, but the knockdown was not lethal (Fig. 4, E and F).

**pH**<sub>i</sub> Homeostasis in Procyclic Forms Deficient in P-type H<sup>+</sup>-ATPases—To investigate whether TbHA expression inhibition results in an alteration in the pH<sub>i</sub> homeostasis of procyclic forms, we examined the steady-state pH<sub>i</sub> of control and mutant cells deficient in TbHA1,3 and in TbHA2. In uninduced cells, the mean base-line pH<sub>i</sub> of procyclic trypomastigotes in the nominal absence of bicarbonate and at an extracellular pH of 7.4 in standard buffer was 7.61 ± 0.01 (n = 5), a value similar to that reported previously (2). In agreement with the lower effects

![FIGURE 6. pH<sub>i</sub> recovery of acid-loaded procyclic forms.](image)

Acidification was obtained by preincubation of 1.5 × 10<sup>7</sup> cells with 40 mM NH<sub>4</sub>Cl for 15 min and then resuspension in standard buffer at pH 7.4. A, pH<sub>i</sub> recovery of uninduced control (C) and TbHA1,3 mutant cells induced for 4 days (M); B, pH<sub>i</sub> recovery of uninduced control (C) and TbHA2 mutant (M) cells induced for 4 days.

![FIGURE 7. Expression of TbHA in yeast.](image)

A, drop test for the growth of yeast strains in galactose (upper lane, SGAH) or glucose (lower lane, SDAH) medium. In SGAH medium, all strains (except MP625 and RS1002) could potentially express both yeast PMA1 and transfected TbHA genes. In SDAH medium, only the TbHA constructs could be expressed (except in RS1002). Strains RD2011 (expressing TcHA1), RD0511 (expressing TbHA1), RD0522 (expressing TbHA2), RD0533 (expressing TbHA3), control strain MP625 (expressing yeast PMA1 only in galactose medium), and control strain RS1002 (expressing yeast PMA1 in both media) were used. Cells were grown to saturation in galactose medium, and ∼10<sup>7</sup> cells in 5 μl were spotted on agar plates containing medium as indicated. Growth was recorded after 4 days at 30 °C. B, growth of yeast strains in liquid media. Cells were grown to saturation in SGAH medium, and ∼10<sup>7</sup> cells in 5 μl were inoculated into 5 ml of SGAH or SDAH medium. Growth was estimated by measuring the absorbance at 660 nm after 4 days at 30 °C. Data are shown the means ± S.D. of five independent experiments.
observed on growth inhibition (Fig. 4C), procyclic forms obtained after 4 days of induction of the RNAi construct directed against TbHA2 did not show any marked effect and had a similar base-line pH compared with uninduced cells (7.59 ± 0.02, n = 5). In contrast, procyclic forms obtained after 4 days of induction of the RNAi constructs directed against TbHA1 and TbHA3 showed a reduced base-line pH (7.37 ± 0.05, n = 5).

To further investigate the role of the P-type H^+ -ATPases in pH regulation, procyclic forms were acidified to pH 6.6–6.8 using the NH_4Cl prepulse technique (2). Mutant procyclic forms transfected with the RNAi constructs directed against TbHA1 and TbHA3 (Fig. 6A), but not against TbHA2 (Fig. 6B), recovered their pH, more slowly and reached a lower final pH compared with uninduced cells. This was not surprising because the morphological changes were more evident after knockdown of TbHA1 and TbHA3, and the availability of these proton pumps could probably compensate the decrease in TbHA2 expression. Together, these results further confirm the role of P-type H^+ -ATPases in pH regulation in procyclic forms (2).

Functional Rescue of P-type H^+ -ATPase Activity in Yeast PMA1-deficient Mutants—To investigate whether TbHA1, TbHA2, and TbHA3 encode functional proton pumps, we expressed them in mutant yeast (22). The yeast H^+ -ATPase gene PMA1 is essential and rate-limiting for growth (34); therefore, a mutant strain (RS-72) that has PMA1 under the control of a galactose-dependent promoter cannot grow in glucose medium (32, 35). This strain may then be transformed with a yeast multicopy vector carrying a heterologous H^+ -ATPase gene under the control of a constitutive PMA1 promoter. In galactose medium, the transformed yeast strain expresses both PMA1 and the heterologous H^+ -ATPase, whereas in glucose medium, growth is dependent on the heterologous H^+ -ATPase alone. Therefore, three expression vectors were constructed to express the complete forms of TbHA1, TbHA2, and TbHA3 in yeast strain RS-72. We investigated the ability of these strains to grow in glucose and galactose media at the optimal pH, 5.5. The results are shown in Fig. 7. The positive control (strain RS1002 with yeast PMA1 under the control of its own promoter) grew well at pH 5.5 in both media (Fig. 7A). The negative control (strain MP625 with yeast PMA1 under the control of the GAL1 promoter) grew well only in galactose medium (Fig. 7A). Strains RD2011, RD0511, RD0522, and RD0533, expressing TcHA1 (9), TbHA1, TbHA2, and TbHA3, respectively, complemented the yeast H^+ -ATPase (Fig. 7A). The growth properties of strains RD2011, RD0511, RD0522, and RD0533 in liquid medium (Fig. 7B) were comparable with those of RS1002 and MP625 in galactose medium. The control strain MP625 did not grow in glucose medium, and growth supported by TbHA2 (RD0522) and TbHA3 (RD0533) was somewhat lower than in yeast expressing the homologous yeast H^+ -ATPase (RS1002).

Absence of Vanadate-sensitive H^+ -ATPase Activity in Permeabilized Procyclic Forms—Previous studies using permeabilized T. cruzi (37) and Phytomonas francisci (38) cells demonstrated the presence of intracellular vanadate-sensitive (P-type) H^+ -ATPases activities. In the case of T. cruzi, a vanadate-sensitive H^+ -ATPase is localized in its endocytic compartment (15). Because we occasionally detected some intracellular labeling in immunofluorescence experiments using anti-TcHAf antibody (Fig. 3, A and C), we performed experiments with permeabilized T. brucei procyclic forms to rule out the presence of an intracellular H^+ -ATPase (Fig. 8). As reported previously (39), when procyclic trypomastigotes were permeabilized with digitonin, some acidine orange was accumulated and retained in the absence of energy sources (data not shown). Once a steady state of acidine orange accumulation was reached, addition of 1 mM ATP led to additional dye uptake (Fig. 8A). The gradient collapsed completely after addition of 1 \mu M nigericin (Fig. 8A). When the cells were preincubated in the presence of 500 \mu M vanadate, no inhibition was detected (Fig. 8B), whereas when the cells were preincubated with 500 \mu M bafilomycin A_1, dye uptake was almost completely inhibited (Fig. 8C).

Similar experiments with permeabilized bloodstream forms showed that proton transport in permeabilized cells was not inhibited by 100 \mu M vanadate (data not shown). These results indicate the absence of an intracellular vanadate-sensitive (P-type) H^+ -ATPase activity and that most ATP-driven dye uptake occurs through a bafilomycin A_1-sensitive H^+ -ATPase activity (vacuolar H^+ -ATPase) that has been characterized previously (39 – 41).

**DISCUSSION**

We reported previously a major role for a plasma membrane H^+ -ATPase in the regulation of pH and ΔΨ in procyclic stages and a minor role in the regulation of pH and ΔΨ in bloodstream forms (2, 4) of T. brucei. In this work, we have demonstrated that genes encoding proton pumps with homology to P-type H^+ -ATPases are present in the T. brucei genome (TbHA1,
**Molecular Characterization of T. brucei P-type H\(^+\)-ATPases**

*TbHA2* and *TbHA3*. These genes could complement a yeast strain deficient in H\(^+\)-ATPase, providing genetic evidence that they encode functional proton pumps.

Examination of the protein sequences of *TbHA1*, *TbHA2*, and *TbHA3* indicated that they are members of the family of P-type H\(^+\)-ATPases. This is supported by phylogenetic analysis grouping these ATPases with a cluster that includes the *Leishmania* and *T. cruzi* proton pumps (8). As occurs with the *T. cruzi* proton pumps (9), but not with the *L. donovani* proton pumps (42), the *T. brucei* ATPases apparently lack the C-terminal domain present in other P-type H\(^+\)-ATPases that constitutes the nonessential inhibitory domain involved in the regulation of the enzyme by glucose metabolism (43). However, *TbHA2* and *TbHA3*, but not *TbHA1*, *TcHA2*, or the *Leishmania* proteins, possess a C-terminal motif (Pro-Thr-Leu) that is very similar to the C-terminal motif (Tyr-Thr-Val) present in several plant H\(^+\)-ATPases (43). Phosphorylation of the penultimate residue (Thr) of the C-terminal motif of *TbHA2* and *TbHA3*, but not *TbHA1*, *TcHA2*, or the *Leishmania* -ATPases allows the enzyme to form a stable complex with 14-3-3 regulatory proteins, resulting in activation of the enzyme (43).

Stringent genetic validation of putative drug targets is desirable before the rational design of inhibitory compounds intended for chemotherapeutic use is undertaken (44). This study validates *T. brucei* P-type H\(^+\)-ATPases as drug targets through the use of RNAi methods. Traditional methods for generating genetic knock-outs in *T. brucei* are time-consuming. In contrast, RNAi has been shown to be a much simpler and faster method for selective interference of gene expression (33, 45). Unlike gene knock-outs, in which each locus must be individually disrupted, RNAi can inhibit expression of multiple copies of a target gene. Furthermore, RNAi does not even require fully cloned genes, whereas traditional knock-outs generally need at least the 5' and 3'-flanking regions. This feature makes RNAi a powerful tool to assess the function of genes in *T. brucei*. Inhibition of *TbHA* expression in procyclic forms caused morphological alterations (Fig. 5, F and G) and growth inhibition (Fig. 4, A–C). In addition, inhibition of *TbHA1* and *TbHA3* expression, but not *TbHA2* expression, in procyclic forms caused alterations in pH\(_i\) (Fig. 6, A and B), further confirming the role of these proton pumps in the regulation of pH\(_i\), homeostasis (2).

In the bloodstream forms, RNAi of individual proton pumps slowed growth only for the first 24 h and was not lethal. However, this was due to the leakiness of the vector in these forms because the mRNA was not completely eliminated after induction of the cells with tetracycline. This phenomenon also occurred with mutant procyclic forms if sufficient time was given for them to recover.

In agreement with their proposed role in pumping protons out of the cells (2), *TbHA* proteins were found to localize in the plasma membrane of procyclic and bloodstream forms (Figs. 3, A and C; and 5A). Labeling of bloodstream forms was weaker and also present in the flagellum and intracellular structures. Weak intracellular labeling was also detected in procyclic stages (Fig. 3A). This intracellular labeling could be related to the transit of the proton pump through the secretory pathway, as occurs in yeast cells (22). However, no evidence of a functional intracellular P-type H\(^+\)-ATPase activity was detected in both stages (Fig. 8) (data not shown).

In conclusion, our work provides strong evidence that *T. brucei* possesses functional P-type H\(^+\)-ATPases. Because these enzymes do not have a counterpart in mammalian cells (8, 10) and are essential for growth, they are suitable drug targets.

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