Trypanosoma brucei Vacuolar Transporter Chaperone 4 (TbVtc4) is an Acidocalcisome Polyphosphate Kinase Required for In vivo Infection*

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Background: Polyphosphate (polyP) accumulates in an acidic calcium store named the acidocalcisome.

Results: TbVtc4 is an acidocalcisome polyP kinase required for osmoregulation and virulence.

Conclusion: TbVtc4 is a potential drug target in T. brucei

Significance: This is the first demonstration of an essential polyP kinase in trypanosomes.

Polyphosphate (polyP) is an anionic polymer of orthophosphate groups linked by high energy bonds that typically accumulates in acidic, calcium-rich organelles known as acidocalcisomes. PolyP synthesis in eukaryotes was unclear until it was demonstrated that the protein named vacuolar transporter chaperone 4 (Vtc4p) is a long chain polyP kinase that localizes to the yeast vacuole. Here, we report that the Vtc4 ortholog of Trypanosoma brucei (TbVtc4) encodes, in contrast, a short chain polyP kinase that localizes to acidocalcisomes. The subcellular localization of TbVtc4 was demonstrated by fluorescence and electron microscopy of cell lines expressing TbVtc4 in its endogenous locus fused to an epitope tag and by purified polyclonal antibodies against TbVtc4. Recombinant TbVtc4 was expressed in bacteria, and polyP kinase activity was assayed in vitro. The in vitro growth of conditional knockout bloodstream form (BSF) trypanosomes (TbVtc4-KO) was significantly affected relative to the parental cell line. This mutant had reduced polyP kinase activity and short chain polyP content, and was considerably less virulent in mice. The wild-type phenotype was recovered when an ectopic copy of TbVtc4 gene was expressed in the presence of doxycycline. The mutant also exhibited a defect in volume recovery under osmotic stress conditions in vitro, underscoring the relevance of polyP in osmoregulation.

Polyphosphate (polyP) is an inorganic, linear polymer of orthophosphate (P\textsubscript{i}) units linked by phosphoanhydride bonds. PolyP can exist as short (3 to ~300 P\textsubscript{i}) or long chain (~300 to ~1,000 P\textsubscript{i}) polymers, is abundant in nature, and has been conserved during evolution (1, 2). PolyP has been extensively studied in bacteria, where it is involved in several essential functions as DNA...
replication, sporulation, germination, motility, and pathogenesis. Much less is known of the functions of polyP in eukaryotes (1, 2). The recent discoveries that polyP can be released from some mammalian cells such as blood platelets (3) and mast cells (4) and has potent modulatory activity on blood coagulation (5) and inflammation (6), have renewed interest in this polymer. Interestingly, polyP with chain lengths characteristic of microorganisms modulates coagulation and inflammation differently than polyP with chain lengths typically found in mammalian cells (7).

In many organisms polyP is mobilized primarily by the synthetic activity of polyP kinases and degradation by endo- and exopolyphosphatases, respectively. A few genes encoding exopolyphosphatases (8-11) and endopolyphosphatases (12) have been described in eukaryotes. Recently, the first eukaryotic enzyme involved in synthesis and translocation of polyP, Saccharomyces cerevisiae vacuolar transporter chaperone 4 (ScVtc4p), was identified (13). The Vtc complex consists of four proteins (Vtc1-4) that form hetero-oligomeric complexes and are able to synthesize and transfer polyP into the vacuole, as well as impacting membrane trafficking and vacuole fusion (14-16). Vtc4 forms the catalytic core of the complex, although null mutations of each of the Vtc proteins result in reduced accumulation of polyP. Vtc proteins are present in fungi, algae, trypanosomatids, and apicomplexan parasites but are absent in mammalian cells.

In many cells short and long chain polyP accumulate in acidocalcisomes, acidic calcium stores (17) where polyP is complexed with several cations (18, 19). These organelles were first described in Trypanosoma brucei (20) but later identified in a broad range of organisms from bacterial to human cells (18), and are involved in Ca$^{2+}$ signaling as inferred by the presence in them of an inositol 1,4,5-trisphosphate receptor (21). T. brucei belongs to the group of trypanosomes that causes Human African Trypanosomiasis (HAT, also known as sleeping sickness), an endemic disease of Sub-Saharan Africa. There is no vaccine available for this disease and chemotherapy also remains unsatisfactory, especially for advanced cases when a neurological phase has been reached and the disease becomes potentially fatal.

Previous work has shown that polyP has a critical role in survival of trypanosomes under sharp environmental changes, including osmotic stress (22-24). This resistance to osmotic stress is essential for digenetic trypanosomatids as they encounter drastic osmotic changes in both the insect vectors and vertebrate hosts (23, 25, 26). Regulation of cell volume is, in addition, a homeostatic process needed at all times by all cells. PolyP hydrolysis occurs during hyposmotic stress of trypanosomes (25), probably increasing the osmotic pressure of the acidocalcisomes and facilitating water movement. On the other hand, an increase in long chain polyP levels has been observed in T. cruzi during hyperosmotic stress (22, 23). This latter work suggested that polyP could play an important role at the early stages of hyperosmotic stress response by sequestering ions into the acidocalcisomes to reduce the ionic strength of the cytosol (23).

Homologs of S. cerevisiae Vtc1 and Vtc4 genes are present in the genome of T. brucei. TbVtc1, a protein present in T. brucei acidocalcisomes (27), is essential for polyP synthesis and acidocalcisome biogenesis. However, this protein does not have polyP kinase domain or PPK activity. An ScVtc4p homolog (TbVtc4) was detected in a proteomic analysis of T. brucei acidocalcisomes (Huang, G., Ulrich, P.N., Johnson, D., S.N.J. Moreno, Orlando, R., and Docampo, R., unpublished data). In the present study we investigated the role of this enzyme by biochemical and genetic approaches, elucidating important aspects of its physiological role in T. brucei, where polyP seems to be essential for parasite survival in the mammalian host. Since Vtc4 proteins are absent in vertebrates, we propose this enzyme as a potential target for drug development and disease control.

**EXPERIMENTAL PROCEDURES**
Culture Methods—Cultivation of 29-13 procyclic form (PCF) (28) and single marker bloodstream form (BSF) (29) trypanosomes derived from *T. brucei* Lister strain 427 was carried out as previously described (30). Cell growth was followed using a Beckman® Coulter Z1 Dual Cell and Particle Counter.

Chemicals and reagents—TRIzol reagent, MagicMedia, Taq polymerase, Benchmark Protein ladder, Alexa-conjugated secondary antibodies, and *Escherichia coli* BL21 Codon Plus (DE3)-RIPL were purchased from Invitrogen (Carlsbad, CA). Vector pET32 Ek/LIC, Benzonase®, Nuclease, anti-Histidine tag antibodies, and S-protein HRP conjugate were from Novagen (EMD Millipore, Billerica, MA). [α,32P]dCTP (3,000 Ci/mmoll) and [γ,32P]ATP (3,000 Ci/mmoll) were from Perkin Elmer (Waltham, MA). Rabbit and mouse antibodies against *T. brucei* vacuolar H+-pyrophosphatase (TbVP1) (31) were a gift from Dr. Norbert Bakalara (Ecole Nationale Supérieure de Chimie de Montpellier, Montpellier, France). Anti-HA high affinity rat monoclonal antibody (clone 3F10) was purchased from Roche (Roche Applied Science, Mannheim, Germany). The pMOTag4H vector (32) was a gift from Dr. Thomas Seebeck (University of Bern, Bern, Switzerland). PD-10 desalting columns were from Amersham Biosciences (GE Healthcare Life Sciences, Piscataway, NJ). Pierce ECL Western blotting substrate and Pierce BCA Protein Assay Reagent were from Thermo Fisher Scientific Inc. (Rockford, IL). Zeta-Probe GT Genomic Testing blotting and nitrocellulose membranes were from Bio-Rad (Hercules, CA). AMAXA Human T-cell Nucleofector kit was purchased from Lonza (Germany). Prime-a Gene Labeling System was from Promega (Madison, WI). QIAprep Spin Miniprep and Midiprep kits, QIAquick gel extraction kit and MinElute PCR purification kit were from Qiagen (Valencia, CA). Fluorimetric ADP Assay Kit was from PhosphoWorks (AAT Bioquest, Inc., Sunnyvale, CA). The primers were purchased from Integrated DNA Technologies (Coralville, IA). Antibiotics and all other reagents of analytical grade were from Sigma (St. Louis, MO).

Sequence analysis—The analysis of *TbVtc4* sequence (gene ID Tb11.01.4040) was performed using DNA MAN software (version 7.212, Lynnon Corporation, Quebec, Canada) for alignments, the basic local alignment search tool (BLAST) for searching homologous sequences, Motif Scan algorithm for prediction of functional domains (33), and TopPred algorithm for the prediction of transmembrane domains (34). General information available for this sequence was obtained from TriTrypDB (35).

Gene cloning and protein heterologous expression—DNA sequence corresponding to *TbVtc4* catalytic core (nucleotides 595-1518 of the *TbVtc4* open reading frame, amino acids 199-506 of the full-length protein) was PCR-amplified from *T. brucei* 29-13 strain gDNA (forward primer: 5'-GACGACGACAAAGATACCTTGTTGTA CCGTGGG-3', reverse primer: 5'-GAGGAGAAGC CCGGTGTGGAAGCGCGAATGTCAA-3') and ligation-independent cloned into vector pET32 Ek/LIC for heterologous expression in bacteria. The sequence of several recombinant clones was verified and they were transformed by heat shock into *E. coli* BL21 Codon Plus (DE3)-RIPL chemically competent cells. Induction of *TbVtc4*199-506 expression was performed with MagicMedia following manufacturer’s dual temperature protocol to avoid aggregation of protein in inclusion bodies for purification under native conditions. Protein expression was alternatively induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in LB broth for 3 h at 37 °C for purification under denaturing conditions.

The catalytic domain of ScVtc4p [aa 189-480, (13)] was amplified from yeast gDNA using standard PCR protocols (forward primer: 5'-GAG CTCAAGGGAAGCAACAAATTTC-3', reverse primer: 5'-CACGGTGTACCGGAGTTAACCACAT G-3') and Pfu Ultra HF (Stratagene). The fragment was cloned with a TOPO-TA cloning kit, verified by sequencing, and ligated into the expression vector pQE-2 (Qiagen) using SaeI and PmlI sites that added to the 5' and 3' primers,
An essential polyphosphate kinase in T. brucei

respectively. *E. coli* (BL21 Codon Plus DE3 RIPL, Stratagene) were transformed with ScVtc4<sub>189-480</sub>/pQE-2. Histidine-tagged ScVtc4<sub>189-480</sub> was induced overnight at 25 °C with 0.5 M IPTG, isolated by metal-ion affinity chromatography, and desalted on a HiTrap column (GE Life Sciences) with 25 mM tris, 200 mM NaCl, 2 mM DTT (13).

**Purification of recombinant TbVtc4 catalytic core under native conditions**-Cell pellets from 200 mL culture of recombinant *E. coli* BL21 expressing TbVtc4<sub>199-506</sub> grown in MagicMedia were resuspended and incubated for 30 min on ice in 20 ml cold lysis buffer: 50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, 10 mM imidazole, 0.1% Triton X-100, 0.1 mg/mL lysozyme, 25 U/mL Benzonase<sup>®</sup> Nuclease and protease inhibitor cocktail (Sigma) to get a clarified crude protein extract that was kept on ice and used for immediate purification of recombinant TbVtc4<sub>199-506</sub>. Protein purification was performed at 4 °C using HIS-Select<sup>®</sup> Cartridge (Sigma), an immobilized nickel-ion affinity chromatography, following manufacturer’s protocol for histidine-tagged protein purification under native conditions. One mL fractions were eluted (elution buffer: 50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, 250 mM imidazole) and buffer exchange was performed immediately using PD-10 desalting columns to finally obtain the protein in assay buffer (20 mM Heps, pH 6.5). All purification steps were verified by SDS-PAGE and western blot analyses using anti-Histidine tag commercial antibodies or S-protein HRP conjugate. For antibody production, recombinant TbVtc4<sub>199-506</sub> was purified under denaturing conditions using HIS-Select<sup>®</sup> Cartridge (Sigma).

**Antibody production**-Polyclonal antibodies anti-TbVtc4 were generated in a guinea pig by Covance (Princeton, NJ) against a synthetic peptide (CSRSRRVYARRKIRYDDRRG) that corresponds to a conserved hydrophilic region located between the second and the third predicted transmembrane domains of TbVtc4 amino acid sequence. In addition, polyclonal antibodies anti-TbVtc4 were produced in mice using recombinant TbVtc4<sub>199-506</sub> as antigen. These antibodies were generated at the Monoclonal Antibody Facility of the College of Veterinary Medicine, University of Georgia (Athens, GA). Final bleeds from five inoculated mice were affinity purified by immunoadsorption to the recombinant protein immobilized on nitrocellulose strips. The adsorbed antibodies were eluted with 0.1 M glycine, pH 2.5, and neutral pH was restored immediately by adding 1 M Tris-HCl buffer, pH 8.0.

**Fluorescence microscopy**-For immunofluorescence assays (IFA), *T. brucei* PCF were centrifuged at 1,000 × g for 10 min at 25 °C, washed twice with PBS, pH 7.4, and fixed with 4% paraformaldehyde in PBS for 1 h on ice. Afterwards, cells were adhered to poly-L-lysine coated coverslips, permeabilized with 0.3% Triton X-100 for 3 min, washed three times and blocked with PBS containing 3% BSA, 1% fish gelatin, 50 mM NH<sub>4</sub>Cl, and 5% goat serum for 1 h. Next, cells were incubated for 1 h at room temperature (RT) with primary antibodies: polyclonal guinea pig anti-TbVtc4 (1:50) or rat anti-HA tag high affinity mAb (Roche, diluted 1:10) and polyclonal rabbit anti-TbVP1 (1:250), as acidocalcisome marker. After washing three times with 3% BSA in PBS (pH 8.0), cells were incubated for 45 min at room temperature in the dark with secondary antibodies: Alexa Fluor 488-conjugated goat anti-guinea pig or anti-rabbit (1:1,000) and Alexa Fluor 546-conjugated goat anti-rabbit or anti-mouse (1:1,500). Then, cells were counterstained with 5 µg/mL DAPI to label nuclei and kinetoplast (mitochondrial DNA). Finally, all preparations were washed again three times with 3% BSA in PBS (pH 8.0) and mounted on glass slides with Fluoromount-G (Southern Biotechnology). Differential interference contrast (DIC) and fluorescence optical images were captured under non saturating conditions and
An essential polyphosphate kinase in T. brucei

identical exposure times using an Olympus IX-71 inverted fluorescence microscope with a Photometrix CoolSnapHQ charge-coupled device (CCD) camera driven by DeltaVision software (Applied Precision).

Electron microscopy—BSF trypanosomes were washed twice in 0.1 M sodium cacodylate buffer, pH 7.4, and fixed for 1 h on ice with 0.1% glutaraldehyde, 4% paraformaldehyde and 0.1 M sodium cacodylate buffer, pH 7.4. Samples were processed for cryo-immunoelectron microscopy at the Molecular Microbiology Imaging Facility, Washington University School of Medicine. HA-fusion protein localization was detected with a polyclonal antibody against HA and anti-rabbit gold conjugated as a secondary antibody. Mouse anti-TbVP1 polyclonal antibodies and anti-mouse gold conjugated secondary antibodies were used.

Enzymatic assays for polyP synthesis-ADP assay. For determination of TbVtc4 and ScVtc4p kinetic parameters the specific activity of the enzyme was assayed using an ADP determination kit (PhosphoWorks™ Fluorimetric ADP Assay Kit, AAT Bioquest, Inc) to quantify the amount of ADP or GDP synthesized during polyP polymerization at different ATP or GTP concentrations. Analysis of ATP conversion by the recombinant catalytic domain of ScVtc4p was carried out in buffer containing 50 mM Tris- HCl (pH 7.5), 150 mM NaCl, 1 mM MnCl₂, and 1 mM ATP, at room temperature using 1 µM ScVtc4p. TbVtc4 polyP kinase activity was assayed in buffer containing 50 mM Hepes (pH 6.5), 150 mM NaCl, and 1 mM MnCl₂, at 37 °C using 1 µM TbVtc4 and 1 mM ATP, or different concentrations of ATP, or GTP. When indicated, 1 mM PPᵢ was included in the reactions. Twenty µL reactions were incubated 1 h at room temperature. Components A and B of ADP/GDP determination kit were added immediately, and after 30 min incubation at room temperature, fluorescence was detected at 540/590 nm excitation/emission ratio in a Molecular Devices plate reader. An ADP (or GDP) standard curve was obtained for quantification purposes. The data fit to a Michaelis-Menten equation and GraphPad Prism software version 5.0 was used for data analysis and determination of $K_m$, $V_{max}$ and $k_{cat}$.

Coupled assay. ATP hydrolysis was also monitored via NADH oxidation enzymatically coupled to the rephosphorylation of produced ADP. NADH concentration was measured optically at 340 nm in buffer containing 150 µM NADH, 0.15 mM phosphoenolpyruvate, 0.1 mg/ml pyruvate kinase, and 0.1 mg/ml lactate dehydrogenase. The reaction was initiated by the addition of 1 mM ATP and the incubations were done under the same conditions described above.

Radioactive assay. To visualize TbVtc4 and ScVtc4p reaction products, newly synthesized polyP chains were detected by autoradiography using $[^{32}P]γ$-ATP as substrate. PolyP was separated by electrophoresis on Tris-borate-EDTA (TBE)-polyacrylamide gels. Reactions were carried out as described above using 1 mM $[^{32}P]γ$-ATP (20 Ci/mmol) in a final volume of 50 µL for 8 h at room temperature and stopped by adding EDTA (final concentration = 1 mM) and 10x sample buffer (2 mg/mL Orange G, 30% glycerol). Before loading the samples, TBE-polyacrylamide gels [0.1 x 16 x 20 cm, 1x TBE, 10% polyacrylamide (19:1 acrylamide/bis-acrylamide), 0.05% tetramethylenediamine, 0.05% (w/w) ammonium persulfate] were pre-run at 200 volts for 30 min in a cold room using a PROTEAN® II xi Cell (Bio-Rad). Thirty µL sample and commercial polyP markers were loaded per well as indicated. Gels were run with a 4 mA constant current for 20 h at 4 °C. Different pH and cation requirements were assayed. Dried gels were exposed to films for at least 48 h at -80 °C for autoradiography.

Molecular constructs for TbVtc4 mutant cell lines—For TbVtc4 knockout construction in BSF trypanosomes, one TbVtc4 allele was knocked out by replacement with a puromycin selectable marker in the single marker line that expresses T7 polymerase and tetracycline repressor maintained by a single G418 resistance marker (29). This puromycin cassette was obtained by PCR using a set of long primers (ultramers) containing 100-120 nucleotides from the 5' and 3' UTRs flanking regions of the TbVtc4 ORF (forward primer: 5'-
GCTGTGTTGTGTTTCTTCTTATTGTTACC
AAAGAAGTACGATAAGGAGAACATATAGT
TGAGAGCAGAAGGAAACAAACACAGAG
TTATTACGTTAGGACCGCCGCCCCCTCAGG-3',
reverse primer: 5'-CGTTAACATAGCAACATAC
AGCACATTACTGACAATCAGCACATTACTGACAA
TTACACAACTATGACAGTTTTTCTCTGAGA
CTAGTTGAG-3'), and pMOTag23M vector as
template (32). To replace the second allele we
first introduced an ectopic copy of the gene
(TbVic4ec) under the control of tet-inducible
promoter and selectable by blastidin resistance
inserted at the ribosomal non-transcribed spacer.
This cassette was constructed amplifying TbVic4
gene by PCR from T. brucei single marker strain
gDNA (forward primer: 5'-CAGTGATCATATGCTCC
CCTAGCCAACATATTTCTCTGTCCGCTCTAGAA
CTAGTTGAG-3', reverse primer: 5'-AG
ATGATCATCAGAAGGTGTCGCTTCCGG
CCTTCAGCAAAGCATG) to clone TbVic4ec into pLEW100v5b1d-BSD
expression vector (a gift from Dr. George Cross,
The Rockefeller University, New York City, NY), a
modified version of the original pLEW100
vector (29). The construct was linearized with
NotI restriction enzyme before cell transfection.
Finally, the second TbVic4 allele was knocked out
by replacement with a phleomycin selectable
marker, while keeping the ectopic copy “on” by
addition of tetracycline to the selection medium.
The phleomycin cassette was also PCR amplified
using a primer set containing 100 nucleotides from
TbVic4 ORF 5’ and 3’ UTRs (forward primer:
5'-GCTGTGGTTGTGTTTCTTCTTATTAT
TGTTTACAAAAAGATGACATAGAAGGAGAACA
TTAGTGTGGAGGCAAGGAGAAGCAACA
AACAGAGTTATAACGTGATGAGCGAACCTTGACC
AGTGCCG-3', reverse primer: 5'-CGTTAACATAG
CAGAATCAGCACTTACTGACAATCAGAC
AACATGTACAGCTTCTTCTCAGTGAAGCAGGAC
CATATTTCTCGCCCTCTCTCAGTACTGCTCT
GCTCCTCGCGCA-3') and pUB39 vector, a
modified version of the original pLEW82 vector
(29), as DNA template. The linear constructs
were used for transfection of BSF trypanosomes
(single marker strain) and selection of stable
resistant clones. Finally, a C-terminal HA-tagged
mutant (TbVic4-HA) was generated using also
ultramers (forward primer: 5'-GTAACGTGAACA
TTGTGATATTAGCCGTATTCTTATAACTGT
TAGTGACAGTGATATGTTCCGGTGACGGCTCT
TGCTCAGGGAAGGACCTTCCGTTACCGGG
CCCCCTCTGAG-3', reverse primer: 5'-CGTTAA
ACATAGCAGAACATACGACATTACTGACAA
TCAACCAACATGACAGTTTTTCTCTGAGA
CTAGTTGAG-3'), and pMOTag4H vector (32) as DNA template to
generate a linear fragment that was used to transfect T. brucei PCF and BSF.

Cell transfections—T. brucei BSF were
transfected as previously described (30) with
some modifications. Briefly, 3 × 10⁷ mid-log
phase parasites (cell density in culture below 1 × 10⁶
cells/mL) were harvested by centrifugation at
1,300 × g for 10 min and resuspended in 100 µL of
AMAXA Human T-cell Nucleofector solution.
Then, 10 µg of NotI-linearized plasmid DNA or
purified PCR product (<10 µL) was added to cells
in 2-mm gap cuvettes. Immediately, one
electroporation pulse was applied using program
X-001 of the AMAXA Nucleofector II
apparatus. Following each transfection, resistant
cells were selected and cloned by limiting
dilutions in HML-9 medium containing 20%
tetracycline-free FBS with appropriate antibiotics
(2.5 µg/mL G418, 0.1 µg/mL puromycin, 2.5
µg/mL blasticidin, 2.5 µg/mL phleomycin or 5.0
µg/mL hygromycin) in 24-well plates. Integration
of the constructs into genomic DNA of each
transfected cell line was verified by PCR and
Southern blot analysis.

For PCF transfection, mid-log phase parasites
cell density around 5 × 10⁶ cells/mL) were
harvested by centrifugation at 1,000 × g for 7
min, washed with cold cytomix (2 mM EGTA, 5
mM MgCl2, 120 mM KCl, 0.5% glucose, 0.15
mM CaCl2, 0.01% BSA, 10 mM K2HPO4 /KH2PO4, 1 mM hypoxanthine, 25 mM Hepes, pH
7.6), and resuspended in 0.5 mL of cytomix at a
cell density of 5 × 10⁷ cells/mL. Then, cells were
mixed with 10 µg of PCR product (<50 µL) in a
0.4 cm electroporation cuvette and subjected to
two pulses (1500 V, 25 µF) in a Bio-Rad Gene
Pulser electroporator. The stable transfectants
were obtained in SDM-79 medium supplemented
with 15% FBS plus the appropriate antibiotic (50 µg/mL hygromycin).

**Southern blot analysis**-Genomic DNA from parental and *TbVtc4* mutant cell lines was extracted as described (36). Two µg gDNA were digested overnight with BamHI and HindIII restriction enzymes. Digestion products were resolved by electrophoresis on a 1% agarose gel in Tris-acetate EDTA (TAE) buffer at 50 V. DNA was transferred from agarose gels onto Zeta-probe blotting membranes (Bio-Rad) by capillarity overnight using 0.4 M NaOH as transfer solution. Membranes were hybridized with a radiolabeled *TbVtc4* probe, generated by PCR (forward primer: 5'-GACGACGACAAAGATACCTTGTGATCCGTGG-3', reverse primer: 5'-GAGGAGAAACCCGGTTGGAACGCAGGTTGCAA-3') and labeled with [α-32P]dCTP using random hexanucleotide primers and the Klenow fragment of DNA polymerase I (Prime-a-Gene labeling system, Promega). Membranes were exposed to films for 24-72 h at -80 °C and developed in dark room.

**Northern blot analysis**-Northern blot analysis was performed as previously described (23). Briefly, total RNA was isolated from BSF using TRI Reagent®. RNA samples were subjected to electrophoresis in 1% agarose gels containing 2.2 M formaldehyde, 20 mM MOPS, pH 7.0, 1 mM EDTA, and 8 mM sodium acetate, transferred to nylon membranes, and hybridized with radiolabeled probes for the *Klenow* fragment of DNA polymerase I (Prime-a-Gene labeling system, Promega). Membranes were exposed to films for 24-72 h at -80 °C and developed in dark room.

**Western blot analysis**-Parental and mutant cell lines were separately harvested. Parasites were washed twice in PBS (PCF) or buffer A with glucose (BAG, 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 50 mM HEPES and 5.5 mM glucose, pH 7.3) for BSF and resuspended in RIPA buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS and 0.1% Triton X-100) plus protease inhibitors (mammalian cells protease inhibitor cocktail (P8340 diluted 1:250, 1 mM EDTA, 1 mM PMSF, 2.5 mM TPCK and 100 µM E64) and Benzonase® Nuclease (25 U/mL culture). Then, cells were incubated for 30 min on ice and 5 rounds of freeze-thaw were applied (5 min on dry ice/ethanol bath, 1 min on 37 °C water bath). Cell lysis was verified under light microscope and protein concentration was determined by BCA protein assay (Pierce). Thirty µg protein from each cell lysate were mixed with 4x Laemmlli sample buffer and analyzed by SDS-PAGE in 10% gels. Separated proteins were transferred onto nitrocellulose membranes (Bio-Rad) using a Bio-Rad transblot apparatus. Membranes were blocked with 5% nonfat dried skim milk in PBST (PBS containing 0.1% [vol/vol] Tween 20) overnight at 4 °C. Next, blots were incubated for 1 h at room temperature with different primary antibodies: polyclonal mouse anti-*TbVtc4* (1:500), rat anti-HA tag mAb (1:100), or with anti-tubulin mAb (1:50,000). After five washes with PBST, blots were incubated with the appropriate secondary antibody: HRP-conjugated goat anti-mouse, or anti-rat IgG (1:15,000) for 1 h at RT. After washing five times with PBST, the immunoblots were visualized using ECL Western Blotting Substrate (Pierce) according to the instructions of the manufacturer.

**Short chain and long chain polyphosphate quantification**-Determination of polyP levels in BSF parental and mutant cell lines was performed as previously described by measuring P<sub>i</sub> release by recombinant yeast expolyphosphatase (37).

**Regulatory volume changes under osmotic stress conditions**-For osmotic stress under constant ionic strength, the following buffers described previously (37, 38), with some modifications were used: isotonic (64 mM NaCl, 4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.53 mM MgCl<sub>2</sub>, 5.5
mM glucose, 5 mM Na-Hepes, pH 7.4, and 150 mM mannitol; 320 ± 5 mOsm, as determined using an Advanced Instruments 3D3 osmometer), hypotonic (the same as isotonic but without mannitol; 160 ± 5 mOsm), and hypertonic (the same as isotonic but with increased mannitol concentration to 1.2 M; 980 ± 5 mOsm). Samples of 1 × 10^7 BSF (single marker strain and TbVtc4-KO +/- tetracycline) were collected, washed with isotonic buffer pre-warmed at 37 °C, and resuspended in 100 µL isotonic buffer. Next, cells were transferred to a 96-well plate and changes in cell volume were followed monitoring absorbance at 550 nm in a plate reader with continuous agitation to avoid decantation of parasites. Osmotic stress was induced after 3 min of absorbance recording as follows: hypotonic stress was induced by addition of 200 µL hypotonic buffer to 100 µL cells in isotonic buffer (final osmolarity: 213 mOsm), hyperosmotic stress was induced by addition of 100 µL hypertonic buffer to 100 µL cells in isotonic buffer (final osmolarity: 650 mOsm), and controls adding 100 and 200 µL isotonic buffer to the cells were carried out in parallel. After inducing osmotic stress, absorbance at 550 nm was recorded for additional 10 min. Cell viability was verified in the microscope after 10 min under osmotic stress.

In vivo studies-To evaluate the infectivity of TbVtc4-KO BSF trypanosomes, the cells were cultivated for 14 days in the absence of tetracycline. Exponentially growing cells (single marker and TbVtc4-KO +/- tetracycline) were washed once in HMI-9 medium without selectable drugs and resuspended in the same medium. Eight-week-old Balb/c mice (5 per group) were infected with a single intraperitoneal injection of 2 × 10^4 BSF trypanosomes in 0.2 ml of HMI-9 medium. Mice were given either normal water or water containing 200 µg/mL doxycycline in a 5% sucrose solution (30, 31). The drinking water with or without doxycycline was provided 3 days before infection and exchanged every 2–3 days, continuing throughout the 30-days period. Animals were fed ad libitum on standard chow. Parasitemia levels were monitored 1–2 times/week during the whole experiment (39). Mice were euthanized upon attaining a parasite density over 1 x 10^8 cells/ml. This study was carried out in strict accordance with the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

Statistical analyses-For all experiments, results were expressed as mean values of three independent experiments ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism software version 5.0. Comparison of polyP kinase activity and short and long chain polyP content in different cell lines was performed by Student’s t test with a significance level of 0.05. Comparison of changes in cell volume after induction of osmotic stress in different cell lines was done by Bonferroni’s multiple comparison a posteriori test of one-way ANOVA at all time points after induction of osmotic stress (significance level = 0.05). In this way, the pattern of response to osmotic stress of all cell lines was analyzed during the entire period of observation.

RESULTS

TbVtc4 sequence analysis-TbVtc4 amino acid sequence (NCBI Reference Sequence: XP_829284.1; TriTrypDB sequence: Tb11.01.4040), was aligned with orthologs from other kinetoplastids and also with ScVtc4p (not shown). TbVtc4 sequence shares 72.2%, 66.9% and 23.3% amino acid identity with T. cruzi Vtc4 (TcCLB.511127.100), Leishmania major Vtc4 (LmjF09.0220) and ScVtc4p (AAP21767), respectively. The ORF of TbVtc4 encodes a protein of 793 amino acids with a predicted molecular mass of 91.3 kDa. TbVtc4 has a VTC domain at the hydrophilic region (aa 210-482) and three predicted transmembrane domains (TMDs), one putative (aa 476-496) and two certain (aa 716-738 and aa 759-781). The
catalytic domain (VTC) is highly conserved (61.5% identity) among these four orthologs.

*TbVtc4* localizes to acidocalcisomes—To establish the localization of *TbVtc4*, an in situ tagging technique was used. We designed the tagged gene to remain under wild-type regulation, avoiding the pitfall of overexpression and consequently increased risk of abnormal distribution of the tagged protein. The linear epitope-tagging cassette was transfected into procyclic (PCF) and bloodstream form (BSF) trypanosomes, where it integrated into the original *TbVtc4* locus via homologous recombination. *TbVtc4* co-localized with the vacuolar proton pyrophosphatase (*TbVP1*), an acidocalcisomal marker (31, 40), in both PCF (Fig. 1A) and BSF trypanosomes (Fig. 1C), as detected by immunofluorescence (Fig. 1A) and immunoelectron microscopy (Fig. 1C), respectively. Western blot analysis confirmed the expression of the tagged protein of the expected size and showed that *TbVtc4* expression levels were higher in PCF (Fig. 1D). Similar colocalization results were observed in PCF using affinity-purified, polyclonal antibodies against synthetic peptide fragment from *TbVtc4* (aa 740-759, Fig. 1B).

*TbVtc4* synthesizes short chain polyP and requires divalent cations but not pyrophosphate—To characterize the enzymatic activity of *TbVtc4* we expressed its catalytic domain (*TbVtc4*199-506) as a fusion protein with an N-terminal polyhistidine tag. In Fig. 1E, lane r*TbVtc4* shows that the recombinant protein (including the histag) appears as a strong single band with a molecular mass comparable to the predicted molecular mass (53.4 kDa). The catalytic core of the *S. cerevisiae* enzyme (ScVtc4p189-480) (13) was used as control. We tested the activity of *TbVtc4* with ATP or GTP (Fig. 2A and 2B, respectively). ScVtc4p was assayed with ATP in the absence and presence of PPi (Figs. 2C and 2D, respectively). *TbVtc4* has a higher affinity for ATP than ScVtc4p (Table 1) in the absence of PPi (*TbVtc4* *Km* = 54.8 ± 7.3 μM vs ScVtc4p *Km* = 261.2 ± 48.6 μM). However, the yeast enzyme is much more efficient than *TbVtc4* (ScVtc4p *kcat/Km* = 9.3 x 10^3 s^-1 M^-1 vs *TbVtc4* *kcat/Km* = 3.1 x 10^3 s^-1 M^-1). The presence of PPi did not “prime” or stimulate *TbVtc4* activity (see also Fig. 3C below), as was observed with the yeast enzyme (13). On the other hand, the reported priming effect of PPi on the polyP kinase activity of ScVtc4p was confirmed by the increase in its efficiency in the presence of PPi with a *kcat/Km* ratio of 1.7 x 10^4 s^-1 M^-1 (Table 1).

PolyP produced by *TbVtc4* reactions was visualized using [32P]γ-ATP as substrate followed by Tris-borate-EDTA polyacrylamide gel electrophoresis (TBE-PAGE). *TbVtc4* requires divalent cations, preferentially Mg^{2+}, Mn^{2+} or Zn^{2+} (Figs. 3A and 3D) and an acidic pH (Figs. 3B and 3E) for optimal activity. The increased activity of *TbVtc4* at acidic pH was corroborated using a different method based on a coupled assay that generates NADH upon polyP production. In contrast to *TbVtc4* (optimal pH = 6.0), ScVtc4p exhibited pH optima at 6.0 and 7.5 (Fig. 3F). Finally, TBE-PAGE/autoradiography demonstrated that polyP chains synthesized by *TbVtc4* catalytic core are much shorter (~100-300 P_i residues) than those produced by ScVtc4p (~750 P_i residues) (Fig. 3C), and *TbVtc4* activity was inhibited in the presence of PPi (Fig. 3C).

Reduced expression of *TbVtc4* in BSF trypanosomes results in decreased polyP kinase activity and short chain polyP content—Previous studies demonstrated that polyP is important for trypanosome growth and osmoregulation in trypanosomes (22, 23, 27, 30, 31, 37), but the length of the polyP responsible for these roles was not investigated. To investigate whether the short chain polyP synthesized by *TbVtc4* is involved in these processes we analyzed the phenotypic changes of BSF trypanosomes with a conditional knockout (KO) of *TbVtc4*. In these cells, we replaced both *TbVtc4* alleles with drug resistance genes, but, since *TbVtc4* could be required for growth, we introduced an ectopic copy of the *TbVtc4* gene whose expression depended on presence of tetracycline or doxycycline in the culture medium (Fig. 4A). The genotype of the mutant cell line was verified by Southern blot analysis (Fig. 4B). Levels of mRNA
in the presence or absence of tetracycline were analyzed by Northern blot (Fig. 4C). As expected, there was a decrease in TbVtc4 mRNA levels in the absence of tetracycline for the KO cell line. In the presence of tetracycline, TbVtc4 mRNA levels of the KO mutant were normal because of the ectopic gene expression. The expression level of TbVtc4 in these mutants was confirmed by western blot analysis using mouse polyclonal antibodies against TbVtc4 (Fig. 4D).

The in vitro growth rate of the mutant cell line in the absence of tetracycline was monitored during 2 weeks and compared to that of the parental single marker strain (WT). The growth rate of TbVtc4-KO BSF progressively decreased relative to the parental cell line. TbVtc4-KO BSF partially recovered as escape mutants arose after 14 days (Fig. 5A). PolyP kinase activity after two weeks of withdrawal of tetracycline was significantly decreased in total cell lysates (Fig. 5B). Reduced activity was accompanied by a 35% decrease in short chain polyP (Fig. 5C), but no significant changes in long chain polyP levels (Fig. 5D). In summary, our results indicate that disruption of TbVtc4 in BSF trypanosomes decreases polyP kinase activity and, as a consequence, results in significantly lower levels of short chain polyP.

TbVtc4-KO mutant parasites display an osmoregulatory defect-To investigate the role of TbVtc4 in osmoregulation, we exposed TbVtc4-deficient BSF trypanosomes to hyposmotic and hyperosmotic conditions and evaluated changes of cell volume with time. These parasites showed a defect in the ability to recover cell volume (a process known as Regulatory Volume Decrease or RVD (41)) during hyposmotic stress when compared with parental (WT) and complemented (+tet) cell lines (Fig. 6A). Loss of water was also more pronounced in these parasites during hyperosmotic treatment compared to the single marker cell line. This defect in hyperosmotic response was overcome when we induced the expression of an ectopic copy of the gene (Fig. 6B).

TbVtc4 is required for effective in vivo infection-We tested the infectivity of the TbVtc4-KO mutants in vivo using a mouse model and we found that the mutant cells were considerably less virulent in mice. Once again, the phenotype reverted when an ectopic copy of TbVtc4 gene was induced by doxycycline (Fig. 7A). It is important to mention that TbVtc4-KO (-dox) BSF trypanosomes were able to infect mice as verified by detection of parasites in blood at day 3-4 post-infection. However, the amount of parasites was significantly lower than that observed in the control groups, and, one week post-infection, TbVtc4-KO (-dox) BSF trypanosomes were no longer detected in blood of surviving animals (Fig. 7B). Thus these parasites were unable to persist in the mammalian host.

Taken together, our data indicate that TbVtc4 plays an important role in the infectivity of BSF trypanosomes and this role is possibly related to their reduced ability to survive under the osmotic stress conditions of the host.

**DISCUSSION**

We report here that TbVtc4 encodes a short chain polyP kinase that localizes to the acidocalcisomes of *T. brucei*. The enzyme is important for osmoregulation, in vitro growth, and infectivity of BSF trypanosomes *in vivo*.

In contrast to the *S. cerevisiae* enzyme, which is a very long chain polyP kinase, TbVtc4 catalyzes production of polyP of about 100-300 P, units and was not activated, but inhibited, by PPi. Despite these differences, we found some characteristics of the enzyme similar to those of ScVtc4p (13). Bivalent cation requirements for both enzymes are slightly different (TbVtc4 metal ion specificity is Mg²⁺ > Mn²⁺ > Zn²⁺ > Co²⁺ > Fe²⁺ > Ni²⁺ > Ca²⁺, and ScVtc4p metal ion specificity is Mn²⁺ > Zn²⁺ > Co²⁺ > Mg²⁺ > Fe²⁺ > Ni²⁺). It is important to mention that the acidocalcisome environment of TbVtc4 is rich in zinc, magnesium and calcium (42). Both enzymes can catalyze polyP synthesis at acidic pH. TbVtc4 activity is highest at a pH of 6.0, but ScVtc4p exhibits two optimal pH values (6.0 and 7.5). Although these enzymes are located in the membrane of acidic calcium stores (the
Acidocalcisome and the yeast vacuole, respectively), their catalytic domains are facing the cytosol. It is possible that the microenvironment close to the outer leaflet of the acidocalcisome and vacuolar membranes has a lower pH due to the presence of Na+/H+ exchangers that move protons out from the acidocalcisome (40) and vacuole lumen (43).

A significant decrease in the level of short chain polyP was observed in the knockout BSF trypanosomes. However, a large reservoir was still present after parasites had been cultured for two weeks without tetracycline. The persistence of short chain polyP could be due to known slow turnover of polyP (44) but more likely arises from the methodological limitations to distinguish between abundant forms of very short polyP (polyP₃, polyP₄, and polyP₅) (45), and medium size polyP (100-300 Pi), which are the main product of TbVtc4. The synthetic mechanism of very short polyP is unknown. Nevertheless, our results suggest that medium size polyP is important for osmoregulation and viability. Although previous reports have shown that polyP has important roles in growth and osmoregulation in trypanosomes (22, 23, 27, 30, 31, 37) the length of the polyP responsible for these roles was not investigated. In this work we report that polyP of 100-300 P_i units, which is synthesized by TbVtc4, is required for regulatory volume decrease (RVD) during hyposmotic stress and also relevant for the response of the parasites to hyperosmotic stress conditions. Ability of these parasites to overcome such drastic changes in osmolality is critical from their survival in the mammalian host. BSF trypanosomes in humans must be able to resist osmolality as high as 1,400 mOsm when passing through the renal medulla and rapidly accommodate a return to the isosmotic environment (300 mOsm) of the general circulation (46). Our results demonstrate that depletion of TbVtc4 in BSF trypanosomes leads to defective osmoregulation and infectivity. Both phenotypes are rescued with expression of an ectopic copy of the gene. A possible explanation for these observations is that mutant parasites, with lower short chain polyP levels, are not able to overcome the dramatic cell volume stresses of mammalian renal circulation, and consequently cannot establish an infection. Alternatively, the increased osmotic sensitivity of TbVtc4-deficient trypanosomes weakens the parasites and makes them unable to evade the host immune response.

Long chain polyP is present in trypanosomes (22), but TbVtc4 is not able to synthesize these polymers, therefore other enzyme(s) could be involved in this process. We measured activity of the catalytic region of TbVtc4 because our attempts to recombinantly express the full-length protein in soluble form were unsuccessful. It is possible that the native enzyme can synthesize long chain polyP. However, this is not likely as only short chain polyP synthesis was affected in knockout parasites.

The palmitoyl proteome of T. brucei has recently been reported (47). Both TbVtc1 and TbVtc4 were reported to be palmitoylated. However, our attempts to demonstrate palmitoylation of TbVtc4 in PCF following established protocols (48, 49) were unsuccessful (data not shown).

In summary, the essentiality of TbVtc4 for growth and establishment of an efficient infection suggest that this enzyme is a potential drug target and that it would be possible to develop inhibitors.

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REFERENCES
An essential polyphosphate kinase in T. brucei


**FOOTNOTES**
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FIGURE LEGENDS

FIGURE 1. Localization of TbVtc4 in PCF and BSF trypanosomes. TbVtc4 co-localizes with TbVP1 in acidocalcisomes of PCF (A, B). TbVtc4 was detected with monoclonal anti-HA antibodies in trypanosomes expressing TbVtc4- HA (A) or with polyclonal anti-TbVtc4 antibodies in wild type trypanosomes (B) (green), and co-localized with antibodies against TbVP1 (red). Merge shows co-localization in yellow. Bars = 10 µm. C, TbVtc4-HA fusion protein was also detected in BSF trypanosomes with anti-HA antibodies and gold-conjugate anti-mouse secondary antibody (18 nm) and co-localized with antibodies against TbVP1 and gold-conjugated anti-rabbit antibody (12 nm). Acidocalcisomes (Ac) and kinetoplast (k) are indicated. Bar = 100 nm. D, Western blot analysis in BSF and PCF wild type (WT) and endogenously tagged parasites (TbVtc4) using monoclonal anti-HA antibodies. Anti-tubulin antibodies were used as loading control. Molecular weights are shown on the left. E, Recombinant catalytic region of TbVtc4 (aa 199-506) affinity purified from E. coli (Lane rTbVTC4). Lane MW, protein molecular mass standards (kDa). The 10% SDS-PAGE gel was stained with Coomassie Brilliant Blue. A single protein band corresponds to rTbVtc4.

FIGURE 2. Recombinant polyP kinase activity as a function of substrate concentration. TbVtc4 polyP kinase activity was assayed in buffer containing 50 mM Hepes (pH 6.5), 150 mM NaCl, 1 mM MnCl2, and different concentrations of ATP (A) or GTP (B) at room temperature, using 1 µM TbVtc4. ScVtc4p polyP kinase activity was assayed in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MnCl2, and different concentrations of ATP at room temperature, using 1 µM ScVtc4p in the absence (C) and presence (D) of 1 mM PPi. Other experimental conditions were as described under Experimental Procedures. Values are means ± SD of three independent experiments. Error bars are smaller than the symbols used for some data points.

FIGURE 3. TBE-PAGE analysis of polyP produced by TbVtc4 and ScVtc4p catalytic cores. PolyP synthesized recombinant TbVtc4 and ScVtc4p using [γ-32P] ATP as substrate was analyzed by TBE-PAGE. A, Autoradiography of TbVtc4 reaction products in the presence of different cations. B, Autoradiography of TbVtc4 reaction products at different pH. C, Autoradiography of TbVtc4 (Tb, in the presence of Mn2+, pH 6.0) and ScVtc4p (Sc, in the presence of Mn2+, pH 7.5) reaction products in the presence (+) or absence (-) of PPi. Lanes at the left side of each autoradiography in A-C show polyP of 25, 75 and 750 phosphate units loaded on the same gel and stained with toluidine blue. D, E, Results from 4 (D) and 3 (E) independent experiments similar to those shown in (A) and (B) were quantified by densitometry using SpotDenso tool of AlphalmageR gel documentation system (Proteinsimple, Santa Clara, CA), then normalized and plotted. F, Optimal pH of recombinant TbVtc4 and ScVtc4p was confirmed by an alternative method. Coupling the reaction to lactate synthesis from phosphoenolpyruvate allows the re-generation of ATP to the Vtc4 reaction. Absorbance (340 nm)
An essential polyphosphate kinase in *T. brucei*

turnover indicated NADH consumption during the coupled reaction and was used for measuring poly P kinase (PPK) activity of affinity purified recombinant Vtc4s. Results are expressed in relative activity units at different pHs. Values are means ± SD of three independent experiments.

**FIGURE 4. Generation of TbVtc4 cKO cell line.** A, Schematic representation of the strategy used for the generation of a stable *TbVtc4* conditional knockout mutant in *T. brucei* BSF. i) One allele of *TbVtc4* was replaced with the puromycin-resistance gene (*PAC*) by homologous recombination, generating the *TbVtc4* SKO cell line; ii) An ectopic *TbVtc4* cassette under the control of the tetracycline-inducible PARP promoter and selectable by blasidzin resistance (*BSD*), was inserted at the ribosomal non-transcribed spacer (*rDNA*), generating the *TbVtc4* cSKO cell line. This cassette was constructed using pLEW100v5b1d-BSD expression vector; iii) while keeping induced the expression of the ectopic *TbVtc4*, the second allele of the gene was replaced with a phleomycin resistance gene (*PHLEO*) by homologous recombination, resulting in conditional knockout cell line *TbVtc4* cKO. B, Southern blot analysis of parental cell line (Single Marker, WT), overexpressing (OE), single knockout (SKO), complemented single knockout (cSKO) and complemented double KO (cKO). C, Northern blot analysis of wild type (WT) and *TbVtc4*-KO mutant (cKO) in the absence or presence of tetracycline (± Tet), using a *Vtc4* probe. A tubulin probe was used as loading control. D, Western blot analysis of *TbVtc4* cKO mutant cell line in the absence or presence of tetracycline (± Tet), using polyclonal antibodies anti-TbVtc4 and anti-tubulin antibodies as a loading control. Affinity purified recombinant *TbVtc4*199-506 (rTbVtc4) was included as control. Molecular weights are shown on the left side of the blot.

**FIGURE 5. Effect of inhibition of *TbVtc4* expression on cell growth, PPK activity, and short and long chain polyP levels.** A, *In vitro* growth of single marker BSF (WT, full circles, blue) and *TbVtc4* conditional knockout parasites (*TbVtc4*-KO, full squares, red). B, Lysates from *TbVtc4*-KO BSF showed a ~8-fold lower polyP kinase activity than those from single marker BSF trypanosomes (WT). C, D. Extracts from *TbVtc4*-KO BSF trypanosomes showed a ~35% reduction in short chain polyP content (*C*) with no significant changes in long chain polyP content (*D*), as compared to the parental cell line (WT). Values are means ± SD of three different experiments. *Differences are statistically significant as compared to respective controls, p < 0.05 (Student’s t test).

**FIGURE 6. Effect of inhibition of *TbVtc4* expression on the response of BSF trypanosomes to hyposmotic and hyperosmotic stresses.** The same amount of single marker [WT, blue], *TbVtc4* knockout [cKO (-Tet), red], and *TbVtc4* complemented knockout [cKO (+Tet), green] BSF trypanosomes were suspended in isotonic buffer. *TbVtc4*-KO BSF trypanosomes [cKO (-Tet)] were cultured in the absence of tetracycline for 14 days before de experiment. The cells were then treated as described under Experimental Procedures and relative changes in cell volume were followed by monitoring the absorbance at 550 nm. A, Changes in cell volume after hyposmotic stress (213 mOsm). B, Changes in cell volume after hyperosmotic stress (650 mOsm). Arrows indicate the time point (3 min) when osmotic stress was induced. A decrease in absorbance corresponds to an increase in cell volume and *vice versa*. Values are means ± SD of three different experiments. Asterisks indicate statistically significant differences between cell line patterns, p < 0.05 (Bonferroni’s multiple comparison “a posteriori” test of one-way ANOVA at all time points after induction of osmotic stress).

**FIGURE 7. Effect of inhibition of *TbVtc4* expression on virulence in mice.** Two experiments involving three groups of 5 mice that were infected with single marker (WT) and *TbVtc4*-KO mutant (cKO) BSF trypanosomes (-dox, +dox), were performed. 200 µg/mL doxycycline was supplied in the drinking water of one group of mice for the induction of an ectopic copy of *TbVtc4* gene in the cKO cell
line (complemented knockout control). Percentage of mice survival (A) and parasitemia levels in the blood of infected mice (B) were monitored during 30 days post-infection. The charts combine results from two independent experiments.
Table 1. Kinetic parameters of purified recombinant Vtc4 catalytic regions from *T. brucei* and *S. cerevisiae* with different substrates.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$V_{\text{max}}$ (µmol min$^{-1}$ mg protein$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$)</th>
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<tr>
<td>TbVtc4</td>
<td>ATP</td>
<td>10.2 ± 0.3</td>
<td>54.8 ± 7.3</td>
<td>3.1 x 10$^3$</td>
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<td>GTP</td>
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<td>63.0 ± 13.0</td>
<td>1.9 x 10$^3$</td>
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<tr>
<td>ScVtc4</td>
<td>ATP</td>
<td>145.0 ± 10.2</td>
<td>261.2 ± 48.6</td>
<td>9.3 x 10$^3$</td>
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<tr>
<td></td>
<td>ATP + PP$_i$</td>
<td>256.6 ± 8.6</td>
<td>158.5 ± 16.5</td>
<td>1.7 x 10$^4$</td>
</tr>
</tbody>
</table>
Figure 2

A. TbVtc4, ATP

B. TbVtc4, GTP

C. ScVtc4p, ATP

D. ScVtc4p, ATP (+PPI)

\[ R^2 = 0.9735 \]

\[ R^2 = 0.9324 \]

\[ R^2 = 0.9570 \]

\[ R^2 = 0.9838 \]
Figure 5

(A) Cell density over time for WT and TbVtc4 KO strains.

(B) PolyP kinase activity in WT and KO strains.

(C) Short chain PolyP levels in WT and KO strains.

(D) Long chain PolyP levels in WT and KO strains.
Figure 7

(A) % Survival vs Days post-infection for WT, cKO (-dox), and cKO (+dox).

(B) Parasitemia (Log cells/mL blood) vs Days post-infection for WT, cKO (+dox), and cKO (-dox).
Trypanosoma brucei Vacuolar Transporter Chaperone 4 (TbVtc4) is an Acidocalcisome Polyphosphate Kinase Required for In vivo Infection
Noelia Lander, Paul N. Ulrich and Roberto Docampo

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