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The Acidocalcisome Vacuolar Transporter Chaperone 4 Catalyzes the Synthesis of Polyphosphate in Insect-stages of *Trypanosoma brucei* and *T. cruzi*

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Abstract

Polyphosphate is a polymer of inorganic phosphate found in both prokaryotes and eukaryotes. Polyphosphate typically accumulates in acidic, calcium-rich organelles known as acidocalcisomes, and recent research demonstrated that vacuolar transporter chaperone 4 catalyzes its synthesis in yeast. The human pathogens *Trypanosoma brucei* and *T. cruzi* possess vacuolar transporter chaperone 4 homologs. We demonstrate that *T. cruzi* vacuolar transporter chaperone 4 localizes to acidocalcisomes of epimastigotes by immunofluorescence and immunoelectron microscopy and that the recombinant catalytic region of the *T. cruzi* enzyme is a polyphosphate kinase. RNA interference of the *T. brucei* enzyme in procyclic form parasites reduced short chain polyphosphate levels and resulted in accumulation of pyrophosphate. These results suggest that this trypanosome enzyme is an important component of a polyphosphate synthase complex that utilizes ATP to synthesize and translocate polyphosphate to acidocalcisomes in insect stages of these parasites.

Keywords

calcium; epimastigotes; procyclic forms

Polyphosphate (polyP) is a polymer of inorganic phosphate (P_i) that ranges in length from three to many hundred residues. PolyP is common to all organisms and has a wide variety of physiological functions including transcriptional regulation (Shiba et al. 1997), virulence of *Salmonella* and *Shigella* (Kim et al. 2002), blood coagulation (Smith et al. 2006), phosphorus storage (Rao et al. 2009), and cell volume regulation (Moreno and Docampo, 2013). Intracellular polyP reservoirs are strongly metachromatic, stain strongly in a reaction with methylene blue-sulfuric acid, and were initially described as metachromatic or volutin granules (Babes, 1895; Meyer, 1904). Significant advances in understanding polyP have been made when volutin granules were identified as dynamic organelles now known as acidocalcisomes (Docampo et al. 2005; Docampo and Moreno, 2011). Concentrations of

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inorganic polyP in acidocalcisomes can be as high as the molar range (in terms of P_i monomers, (Ruiz et al. 2001)).

While enzymes that synthesize polyP in prokaryotes have been described (Ahn and Kornberg, 1990; Zhang et al. 2002), a synthetic pathway of polyP in eukaryotes has only recently been elucidated. Prokaryotic polyP synthesis is catalyzed via two distinct enzymes: polyP kinase 1 (PPK1, (Ahn and Kornberg, 1990) and polyP kinase 2 (PPK2 (Zhang et al. 2002)). *Dictyostelium discoideum* is the only eukaryote with a known homolog to prokaryotic PPK (Zhang et al. 2007) and is unique among eukaryotes in that it can also synthesize polyP through a secondary path that proceeds through a complex of actin-related proteins (Gomez-Garcia and Kornberg, 2004). Evidence from *Saccharomyces cerevisiae* studies suggested that a group of four yeast proteins known as vacuolar transporter chaperones (ScVtc1-4p) is involved in polyP metabolism (Ogawa et al. 2000). Fang et al (Fang et al. 2007) demonstrated that a *T. brucei* ScVtc1p homologue (TbVtc1) is localized to acidocalcisomes and that RNA interference of *TbVtc1* reduces polyP levels. Additionally, a ScVtc2p homologue in *Toxoplasma gondii* is also involved in polyP synthesis (Rooney et al. 2011). Conclusive evidence of the role of Vtc's in polyP synthesis was recently established in a structural, biological and biochemical study of ScVtc4p (Hothorn et al. 2009). ScVtc4p catalyzes polyP synthesis in a β -barrel structure via transfer of phosphate from ATP to a growing polyP chain. Activity of ScVtc4p is Mn^{2+} -dependent and enhanced in the presence of PP_i .

Homologues of ScVtc4p are present in *T. cruzi* (TcCLB.511127.100) and *T. brucei* (Tb927.11.12220), and, in previous work (Lander et al. 2013), we demonstrated that TbVtc4 localizes to acidocalcisomes of procyclic (PCF) and bloodstream (BSF) forms of *T. brucei*. The recombinant catalytic region catalyzes synthesis of short chain polyP and is essential for the growth of *T. brucei* BSF *in vitro* and *in vivo*. However, the enzyme is more abundantly expressed in the PCF of *T. brucei*. We demonstrate here that Vtc4 also localizes to *T. cruzi* acidocalcisomes, is an essential enzyme in *T. brucei* PCF, and is responsible for polyP synthesis in these parasite stages. Currently, no effective and toxicity-free treatments of trypanosomatid infections exist. Given that Vtc genes are absent from the genomes of higher eukaryotes, drugs that target Vtc4 function are promising targets for treatments of Chagas disease and human African trypanosomiasis that do not endanger patients' lives.

MATERIALS AND METHODS

Chemicals and reagents

Superscript III reverse transcriptase, hygromycin, MagicMedia, Taq polymerase, BenchMark Protein Ladder, Alexa-conjugated secondary antibodies, and *Escherichia coli* BL21 Codon Plus (DE3)-RIPL and pCRII Blunt Topo were purchased from Life Technologies (Grand Island, NY). Vector pET32 Ek/LIC, Benzonase® Nuclease, anti-Histidine tag antibodies, and S-protein HRP conjugate were from Novagen (EMD Millipore, Billerica, MA), iQ Sybr Green mix were from Bio-Rad (Hercules, CA), G418 was from Calbiochem (Darmstadt, Germany), Pfu Ultra HF polymerase was from Stratagene (La Jolla, CA), Pierce ECL Western blotting substrate and Pierce BCA Protein Assay Reagent were from Thermo Fisher Scientific Inc. (Rockford, IL), anti-HA high affinity rat monoclonal antibody (clone 3F10) was purchased from Roche (Roche Applied Science, Mannheim, Germany). T4 DNA ligase was from New England Biolabs (Ipswich, MA). Kinase-Glo® Luminescent Assay was from Promega (Madison, WI), phleomycin, protease inhibitor cocktail (Cat #P8840), HIS-Select® cartridges, polyclonal anti- GFP antibody, anti-rabbit gold conjugated secondary antibody, TriReagent, DNase, and yeast pyrophosphatase (ScPPase) were from Sigma (St. Louis, MO), rabbit and mouse antibodies against *T. brucei* vacuolar H^+ -pyrophosphatase (TbVP1) (Lemerrier et al. 2002) were a gift

from Dr. Norbert Bakalara (Ecole Nationale Supérieure de Chimie de Montpellier, Montpellier, France). The pMOTag4H vector (Oberholzer et al. 2006) 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). QIAprep Spin Miniprep and Midiprep kits, QIAquick gel extraction kit and MinElute PCR purification kit were from Qiagen (Valencia, 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).

Cell culture

PCF of *T. brucei* (427 strain) were grown at 28 °C in SDM-79 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) as reported before (Huang et al. 2011). HA-tagged TbVtc4 cell lines were maintained in the presence of 50 µg/ml hygromycin. The PCF RNAi cell line (29-13 parent strain expressing T7 RNA polymerase and tetracycline repressor (Wirtz et al. 1999)) was maintained in SDM-79 medium supplemented with 10% heat inactivated FBS, 15 µg/ml G418, 50 µg/ml hygromycin, and 2.5 µg/ml phleomycin.

T. cruzi epimastigotes (Y strain) were grown at 28 °C in liver infusion tryptose (LIT) medium (Bone and Steinert, 1956) supplemented with 10% heat-inactivated FBS. GFP-expressing cell lines were maintained in medium containing 250 µg/ml G418.

Construct design

Constructs of *Vtc4* genes were subcloned from *T. brucei* (427 and 29-13 strains, details in Table S1) and *T. cruzi* genomic DNA (Y strain) using specific oligonucleotides (Table S1) designed with VectorNTI software (Invitrogen). Pfu Ultra HF polymerase was used to amplify *TcVtc4* and *TbVtc4*. The *TbVtc4* RNAi construct was amplified using primers designed with the RNA-iT server (<http://trypanofan.path.cam.ac.uk/software/RNAit.html>, (Redmond et al. 2003)) and cloned into the tetracycline-inducible RNAi vector p27^{ti}B with dual-inducible T7 promoters (LaCount et al. 2002). Cloned sequences were verified by sequencing (Yale DNA Analysis Facility, Yale University, New Haven, Connecticut; Integrated Biotech Laboratories, University of Georgia, Athens, Georgia). We followed the one-step epitope-tagging protocol reported by Oberholzer et al (Oberholzer et al. 2006) to produce the HA-pMOTag construct for *T. brucei*. *TcVtc4a* was amplified by PCR from genomic DNA of Y strain *T. cruzi* using Pfu Ultra HF polymerase (Agilent). Briefly, we performed a total of 30 cycles at 94 °C for 30 s, 59 °C for 30 s, and an extension step at 72 °C for 1 min. The protocol was initiated with a denaturation step at 94 °C for 2 min and concluded with an extension step of 10 min at 72 °C. The amplicon was ligated into pCRII Blunt Topo and verified by sequencing. *TcVtc4a* was ligated with T4 DNA ligase into pTREX-GFP (de Paulo Martins et al. 2010) a modified version of the original pTREX vector (Vazquez and Levin, 1999), following digestion with XbaI and HindIII.

T. cruzi Y strain epimastigotes (4×10^7 cells, at room temperature, suspended in PBS, pH 7.4 containing 0.1% glucose) were transfected in ice-cold cytomix (25 mM Hepes, 120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄, 2 mM EGTA, 5 mM MgCl₂, 0.5% glucose, 100 µg/ml BSA, 1 mM hypoxanthine, pH 7.6) containing 50 µg of each plasmid construct in 4 mm electroporation cuvettes with 3 pulses (1500 V, 25 µF) delivered by a Gene Pulser II (Bio-rad). Stable cell lines were established and maintained under drug selection with G418 at 250 µg/ml. Until stable cell lines were established, LIT media was supplemented with 20% heat-inactivated FBS.

PCF of *T. brucei* 427 strain (2.5×10^7 cells in room temperature PBS, pH 7.4 containing 0.1% glucose) were transfected in ice-cold cytomix containing 10 µg of each plasmid

construct in 4 mm cuvettes with 2 pulses (1500 V, 25 μ F) with resting on ice for 15 min between pulses. Stable cell lines were established under drug selection with addition of phleomycin (RNAi line, 2.5 μ g/ml) or hygromycin (epitope-tagged line, 50 μ g/ml).

Western blot analysis

Protein was extracted from *T. cruzi* epimastigotes or *T. brucei* PCF for western blot analysis. Five milliliters (*T. cruzi*) or 16 ml (*T. brucei*) of cultured cells were washed twice with PBS (pH 7.4) and resuspended in 100 μ L RIPA buffer (20 mM Tris, pH 7.5; 150 mM NaCl, 1 mM EDTA, 1% SDS, 0.1% Triton-X100) supplemented with 2.5 mM EDTA, 2 mM PMSF, and 1/200 protease inhibitor cocktail (Sigma #P8840) (*T. cruzi*) or 200 μ L RIPA buffer supplemented with 2.5 mM EDTA, 2 mM PMSF, and 1/200 protease inhibitor cocktail (same as above) (*T. brucei*). Genomic DNA was sheared by passage through a tuberculin syringe, and extracts were left on ice to solubilize for 1.5 hours (*T. cruzi*) or 15 min (*T. brucei*). Protein was quantified by BCA assay, and 30 μ g (*T. cruzi*) or 15 μ g (*T. brucei*) were separated on 10% PAGE. For *T. cruzi*, proteins were transferred to nitrocellulose for 1 h at 100 V, and the membrane was blocked overnight at 4° C in PBS-T/5% nonfat milk. The membrane was blotted with polyclonal α -GFP (1:5000) and developed with ECL reagent. For *T. brucei*, western blot analysis was performed in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05 % Tween 20). Membranes were blocked overnight at 4° C in PBS-T containing 5% nonfat dry milk prior to blotting with high affinity α -HA antibody (diluted 1:1500). The blots were developed with ECL reagent.

Immunofluorescence Microscopy

T. cruzi epimastigotes were washed with PBS and fixed with 4% paraformaldehyde/PBS for one hour on ice. Cells were adhered to poly-lysine coated coverslips followed by permeabilization for 3–4 min with 0.3% triton X-100. Permeabilized cells were blocked for 1 h with 3% BSA, 1% fish gelatin, 5% goat serum, 100 mM NH_4Cl in PBS (pH 8). Cells were washed with PBS or PBS/3% BSA and incubated with primary antibody (polyclonal rabbit α -TbVP1, 1:500; monoclonal α -GFP 3E6, 1:400; and/or high affinity rat α -HA, 1:1000) for 1 h. Excess primary antibody was removed from the cells with a series of washes and cells were incubated with secondary antibody conjugated with various Alexa dyes at a 1:2000 dilution for 1 h. Following incubation with secondary antibody, the cells were washed and mounted to slides. DAPI (2.5–5 μ g/ml) was included with the secondary antibody or the mounting medium to stain DNA. Secondary antibody controls were performed as above but in the absence of primary antibody. Differential interference contrast (DIC) and fluorescence optical images were captured under non saturating conditions using an Olympus IX-71 inverted fluorescence microscope with a Photometrix CoolSnapHQ charge-coupled device (CCD) camera driven by DeltaVision software (Applied Precision, Issaquah, WA).

Electron microscopy

T. cruzi epimastigotes overexpressing a TcVtc4-GFP fusion protein were washed twice in 0.1 M sodium cacodylate buffer, pH 7.4, and fixed for 1 h on ice in 0.1 M sodium cacodylate buffer (pH 7.4) containing 0.1% glutaraldehyde and 4% paraformaldehyde. Samples were processed for cryo-immuno-electron microscopy at the Molecular Microbiology Imaging Facility, Washington University School of Medicine. Localization of TcVtc4-GFP was performed with a polyclonal antibody against GFP and anti-rabbit gold conjugated as a secondary antibody.

For imaging whole *T. brucei* PCF, cells were washed with buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 50 mM Hepes, pH 7.3) twice, directly applied to Formvar-coated copper grids, and observed in a Tecnai T20 scanning transmission electron microscope,

operating at 200 kV, using a high angle annular dark field detector. Alternatively, an energy-filtering Zeiss EM 902 electron microscope operating at 80 kV was used. In this case, electron spectroscopic images were recorded at an energy loss of 60 eV using a spectrometer slit width of 20 eV. Determination of morphometric parameters was done as described previously (Fang et al. 2007).

Gene cloning and protein heterologous expression

The DNA sequence corresponding to TcVtc4 catalytic core (nucleotides 595–1518 of the *TcVtc4* open reading frame, amino acids 199–506 of the full-length protein) was PCR-amplified from *T. cruzi* Y strain gDNA (Table S1) and cloned with a ligation-independent protocol into vector pET32 Ek/LIC for heterologous expression in bacteria. The sequences of several recombinant clones were verified, and they were transformed by heat shock into *E. coli* BL21 Codon Plus (DE3)-RIPL chemically competent cells. Induction of TcVtc4_{199–506} expression was performed with MagicMedia following the manufacturer's dual temperature protocol to avoid aggregation of protein in inclusion bodies for purification under native conditions.

Purification of recombinant TcVtc4 catalytic core under native conditions

Recombinant *E. coli* BL21 expressing TcVtc4_{199–506} were grown in 200 ml MagicMedia. Cells were pelleted by centrifugation prior to resuspension and incubation 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® Nuclease and protease inhibitor cocktail for purification of histidine-tagged proteins, 50 μ L g⁻¹ cell paste). Then, cells were subjected to three sonication pulses (40% amplitude, 30 s, on ice) to ensure complete disruption. After centrifugation at 20,000 $\times$ g for 30 min at 4 °C, the supernatant was clarified by passage through nitrocellulose (0.8 μ m) and kept on ice. Recombinant TcVtc4_{199–506} was immediately purified at 4 °C on an immobilized nickel-ion affinity column (His-Select® cartridges) following the manufacturer's protocol. One milliliter fractions were eluted with 50 mM sodium phosphate, pH 8.0; 0.3 M sodium chloride; 250 mM imidazole, and buffer exchange was performed using PD-10 desalting columns to obtain the protein in assay buffer (20 mM Hepes, pH 6.5). Pooling eluted fractions 1–3 and then applying them to a PD-10 column obtained the desalted fraction of purified TcVtc4. All purification steps were verified by SDS-PAGE and western blot analyses using anti-histidine tag commercial antibodies and S-protein HRP conjugate. The lower molecular mass bands recognized by anti-His antibody in fraction E2 were probably due to some degradation, but these bands were not detected in the desalted fraction under the same exposure conditions.

Enzymatic assays for polyP synthesis

For determination of TcVtc4 kinetic parameters, the specific activity of the enzyme was assayed quantifying the ATP consumed during polyP synthesis using the Kinase-Glo® Luminescent Assay in a plate-reader (Synergy™ H1 Hybrid Microplate Reader, Biotek®). TcVtc4 polyP kinase activity was assayed at room temperature using 2 μ M TcVtc4_{199–506} in 50 μ L reactions (50 mM Hepes pH 6.0, 150 mM NaCl, and 1 mM MnCl₂). All reactions were conducted in white, 96-well microplates. ATP (freshly prepared in 50 mM Hepes, pH 7.0) was added to the assay mixture at different concentrations to start the reaction. To determine if PP_i impacts polyP synthesis by TcVtc4_{199–506}, 1 mM PP_i was included in some cases. Negative control reactions without enzyme were included on the same plate. Reactions (50 μ L) were incubated for 1 h at RT. Kinase-Glo Reagent (50 μ L) was then added and the reaction was incubated for additional 10 min before measurement of luminescence. An ATP standard curve was run on the same plate for quantification

purposes. GraphPad Prism software version 5.0 was used for data analysis and determination of K_m , V_{max} and k_{cat} .

RNA Interference

Knockdown of *TbVtc4* was induced with tetracycline in PCF transfectants carrying the RNAi cassette from p2T7^{ti}B. Transcription of the dsRNA construct was induced by addition of 1 µg/ml tetracycline to cultures at a density of 1.15×10^6 cells/ml. Control cultures were always grown alongside for comparison. Every three days or as needed, cell cultures were passed to fresh media to a density of $\sim 1 \times 10^6$ cells mL⁻¹. Experiments were independently replicated on at least 3 different occasions. Knockdown of *TbVtc4* was confirmed using QRT-PCR. Total RNA was isolated from control and induced cultures ($\sim 10^7$ cells per isolation) using TriReagent and treatment with DNase. First strand cDNA was synthesized from 15 µg total RNA using Superscript III reverse transcriptase oligoDT primers. Quantitative PCR was performed in 96 well plates using iQ Sybr Green mix on an iCycler iQ instrument (Bio-Rad, Hercules, CA) using cDNA derived from 30 ng of total RNA. PCR was performed with an initial denaturation at 94° C for 3 min followed by 40 cycles of 94° C for 15 s, 50° C for 20 s, and 72° C for 20 s. Melting profiles were performed from 55–95 °C after each reaction. Alongside *TbVtc4* amplification reactions, triplicate amplifications of *T. brucei* ssRRNA and tubulin genes were included as internal controls. Duplicate non-template controls using each primer set and RNA controls (DNase-treated RNA samples using tubulin primers) were included on each plate. Threshold C_t data was extracted from quantitative PCR data using iCycler iQ software (Bio-Rad, Hercules, CA) and C_t values for *TbVtc4* were normalized to the geometric mean (Vandesompele et al. 2002) of the C_t of *T. brucei* tubulin and ssrRNA genes amplified simultaneously from the same sample.

Polyphosphate extraction and measurement

PP_i and short chain polyP were extracted from PCF *T. brucei* using a protocol modified from previously described methods (Fang et al. 2007). Because short chain polyP extraction yield varies considerably among biological samples, extraction protocols were optimized with respect to the number of each cell type. Briefly, cells (long chain polyP extraction, 2.5×10^7 cells; short chain polyP and PP_i extraction, $\sim 6 \times 10^7$ cells) were washed two or three times with room temperature buffer A containing glucose (BAG) (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose and 50 mM Hepes, pH 7.2), resuspended in 40 µL BAG, and carefully recounted. Approximately 6×10^7 PCF *T. brucei* were resuspended in 100 µL BAG after washing. Two hundred microliters of fresh, ice-cold perchloric acid (0.5 M) were mixed with the cells. Lysed cells were placed on ice for 30 min. Cell debris were removed by centrifugation, and the supernatant was neutralized with a solution of 0.72 M KOH/0.6 M KHCO₃. After 15 min at room temperature, the precipitated salt was removed by centrifugation. Because short chain polyP extracts are unstable, assays of PP_i and short chain polyP were performed within 3 h.

Long chain polyP was extracted from each sample in quadruplicate using glass milk (Qbiogene, Montreal, QC, Canada) as described elsewhere (Ault-Riche et al. 1998). Prior to extraction, PCF parasites were washed and counted as described above for short chain extractions. A sample of GITC-solubilized cells was reserved for protein determination by BCA (Pierce, Thermo Scientific, Rockford, IL) before binding the polyP to glass milk. To minimize variation, long chain polyP extraction protocols were first optimized with respect to cell number. We found that it is necessary to perform quadruplicate extractions of 2.5×10^7 PCF cells from each sample to adequately control variation introduced by extraction methodology (data not shown).

Enzymatic assays of polyP and PP_i were performed in triplicate using recombinant yeast exopolyphosphatase (ScPPX, (Ruiz et al. 2001)) and yeast pyrophosphatase (ScPPase, Sigma, St. Louis, MO) respectively. Assays for short chain polyP were incubated at 35° C for 30 min in 20 mM Tris (pH 7.5), 100 mM ammonium acetate, 5 mM magnesium acetate containing the sample and >50 U ScPPXp. Background phosphate contamination of each sample was measured in triplicate wells not containing ScPPX, and positive control reactions containing a final concentration of 20 μM sodium triphosphate were included on each plate. Reactions were stopped by addition of freshly mixed, malachite green reagent (3 parts 0.045% malachite green and 1 part 4.2% ammonium molybdate/4 M HCl), and absorbance at 660 nm was immediately read on an M2^e microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Release of P_i from PP_i was assayed for 10 min at 35° C in 50 mM Tris (pH 7.5) containing 5 mM MgCl₂ and 0.1 U ScPPase. Background phosphate controls (no ScPPase) and positive control (20–25 μM potassium PP_i) reactions were included in triplicate. P_i was measured with malachite green as described above. On all occasions, standard curves of potassium P_i were included on each plate. P_i released from polyP and PP_i were normalized to reaction yield as determined by positive controls.

RESULTS

Residues essential for yeast Vtc4p activity are conserved in TcVtc4 and TbVtc4

The amino acid sequences of the catalytic regions of TbVtc4 and TcVtc4 (isoforms a and b) were compared with yeast Vtc4p characterized by Hothorn et al (Hothorn et al. 2009) (Fig. 1A). Two sequences of Vtc4 were amplified from *T. cruzi* Y strain, both of which are slightly different than the *T. cruzi* CL strain genomic sequences. The predicted primary structure of TcVtc4a (from now on named as TcVtc4) and TcVtc4b differ from one another only in 3 amino acid residues. TbVtc4 and TcVtc4a/b share 72% identity. While trypanosomatid Vtc4 proteins are only 25% identical to *S. cerevisiae* Vtc4 (GenBank ID: p47075), lysine and arginine residues essential for yeast Vtc4 activity are conserved in both TbVtc4 and TcVtc4a/b. Additionally, key glutamate and serine residues (E426 and S457 in *S. cerevisiae* Vtc4, respectively) are also conserved in the trypanosomatid genes. We chose TcVtc4b for expression of the recombinant catalytic domain and activity assays, as this domain shares a slightly higher identity with the *T. brucei* homolog TbVtc4. Sequences for TcVtc4a, and TcVtc4b were submitted to Genbank as KF745867, and KF745868, respectively.

TcVtc4 localizes to acidocalcisomes

The C-terminus of vacuolar transport chaperone 4 was tagged in *T. cruzi* with GFP. TcVtc4 was overexpressed as a fusion protein in epimastigotes. Western blot analysis confirmed expression of a protein of the expected size (Fig. 1B). Interestingly, this protein is sensitive to denaturing conditions and must not be boiled prior to loading onto polyacrylamide gels. The protein clearly co-localized with the acidocalcisomal marker vacuolar pyrophosphatase (VP1) (Lemerrier et al. 2002) by immunofluorescence analysis (Fig. 2). To further validate this localization we performed cryo-immunoelectron microscopy and demonstrated that TcVtc4a (Fig. 3) clearly occurs in acidocalcisomes and not with any other structure in the cells.

TcVtc4b has polyP kinase activity

To characterize the enzymatic activity of TcVtc4, we expressed its catalytic domain (TcVtc4_{199–506}) as a fusion protein with an N-terminal polyhistidine tag. The recombinant protein was purified using metal-ion affinity chromatography and the fractions were analyzed by SDS-PAGE and western blot (Fig. 4A–C). The recombinant protein (including the his-tag) appears as a strong single band with a molecular mass comparable to the

predicted molecular mass (53.3 kDa, Fig. 4B). We tested polyP kinase activity of TcVtc4 with ATP (Fig. 4D). TcVtc4 has a K_m for ATP of $103.4 \pm 20 \mu\text{M}$ and a V_{max} of $5.1 \pm 0.2 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$. The presence of PP_i did not “prime” or stimulate TcVtc4 activity, as was observed with the yeast enzyme (Hothorn et al. 2009).

TbVtc4 is essential in PCF *T. brucei*

Knockdown of *TbVtc4* mRNA by induction of double-stranded RNAi with tetracycline resulted in growth defects and changes in acidocalcisome morphology and number. Growth defects in PCF developed after 5–6 days of RNAi (Fig. 5A). QRT-PCR of *TbVtc4* from treatment and control cells confirmed that *TbVtc4* RNA levels (normalized to tubulin and ssRNA transcript abundance) were knocked down >70% (Fig. 5B, C).

Additionally, we investigated changes in morphology and number of acidocalcisomes in response to RNAi (Fig. S1A–C and Table S2) by analyzing contrast tuned images obtained by energy-filtering transmission electron microscopy. Comparison of cells from control and induced cultures suggest that mean number of acidocalcisomes dropped by ~2-fold, but individual acidocalcisomes were considerably larger and less circular than those of un-induced cultures (Fig. S1C).

RNAi of *TbVtc4* affects poly P balance

Knockdown of *TbVtc4* transcripts affected cellular polyP and PP_i content (Fig. 6). Short chain polyP fell almost 2-fold ($p < 0.05$, $n = 3$, Mann-Whitney test) over 6 days of RNAi while PP_i nearly doubled ($p < 0.05$, $n = 3$, ANOVA). RNAi of *TbVtc4*, however, did not significantly affect long chain polyP content during the same time frame. When experiments were extended to 9 days, long chain polyP continued to show no significant changes in total concentration ($p > 0.05$, $n = 3$, Mann-Whitney test; data not shown).

DISCUSSION

Bloodstream form (BSF) *Trypanosoma brucei* lacking detectable TbVtc4 through genetic ablation are defective in short chain polyP and exhibit growth defects *in vitro* and *in vivo* (Lander et al. 2013). In the present study, we report that insect forms of both *T. brucei* and *T. cruzi* also express a Vtc4 protein that is able to catalyze polyP synthesis and resides in acidocalcisomes, as detected by immunofluorescence and immunoelectron microscopy. Knockdown of TbVtc4 in procyclic forms (PCF) of *T. brucei* inhibited growth, increased cellular levels of pyrophosphate (PP_i), and decreased levels of short chain polyP. In addition, RNAi of *TbVtc4* in PCF did not affect levels of long chain polyP, as happens after knockout of *TbVtc4* in BSF (Lander et al. 2013). Knockdown of *TbVtc4* in PCF reduced the number of acidocalcisomes and resulted in increased acidocalcisome size relative to wild type cells. The decreased polyP content suggests that ablation of *TbVtc4* shifts the equilibrium of synthesis and degradation of the polyP pool toward hydrolysis. Increased polyP hydrolysis would increase acidocalcisome osmolyte (P_i and PP_i) concentrations and stimulate water entry, as described when polyP is hydrolyzed in trypanosomes subjected to hyposmotic conditions (Ruiz et al. 2001). This is consistent with the increased size of acidocalcisomes that we observed in PCF *T. brucei* whose levels of TbVtc4 were knocked-down by RNAi.

Although short chain polyP decreased by ~50% after 6 days of RNAi, polyP did not disappear completely. PolyP may persist due to residual TbVtc4, or alternative pathways for polyP synthesis that may be present in these parasites. Intracellular polyP is composed of different size classes, and synthesis of very short chain polyP (polyP₃, polyP₄, and polyP₅) which are very abundant in trypanosomes (Moreno et al. 2000) may not be mediated by

TbVtc4. Additionally, the extraction method we used cannot discriminate between very short and medium size polyP.

PolyP plays an important role for resistance of *T. cruzi* to osmotic stresses these parasites experience *in vivo* (Li et al. 2011; Ruiz et al. 2001). The insect stages of this parasite reside in the hindgut of their insect hosts and are subjected to dramatic changes in osmolarity. Following a blood meal, the hindgut osmolality of *T. cruzi* host rises within a day from as low as 300 mOsm to as high as 1,000 mOsm as the blood meal is dehydrated (Kollien and Schaub, 2000). Much less is known about the osmotic conditions in the *tse tse* fly gut, but it is reasonable to speculate that osmolality of the intestine changes as the blood meal is digested.

In summary, Vtc4 is essential for growth of the *T. brucei* PCF, and the *T. cruzi* enzyme is similar to *T. brucei* Vtc4 in its localization and capacity to synthesize polyP. Given that mammalian hosts lack similar enzymes, Vtc4 proteins may be promising drug targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Sequence analysis of Vtc4 proteins and western blot analysis of the tagged TcVtc4
A. Alignment of *TbVtc4* and both *TcVtc4* sequences with the polyP-synthesizing domain of *S. cerevisiae* Vtc4. Secondary structure (predicted by PSIPRED v 2.6, (Jones, 1999; McGuffin et al. 2000)) for *TbVtc4* and *ScVtc4* are shown alongside the alignment in gray and white, respectively. Essential residues of *ScVtc4* are denoted with black triangles and their residue number. Red background highlights essential residues conserved in all Vtc4 sequences. **B.** Western blot analysis of TcVtc4-GFP in an extract from epimastigotes. TcVtc4 was overexpressed with a C-terminal GFP construct and detected with polyclonal α-GFP antibodies.

Figure 2. Immunofluorescence analysis of TcVtc4

A, B. Left panels are differential interference contrast (DIC) images of epimastigotes shown in the right panels. TcVtc4-GFP fusion protein (TcVtc4, *green*) co-localizes with the vacuolar proton pyrophosphatase (VP1, *red*), an acidocalcisome marker, as shown in the Merge images. TcVtc4-GFP was detected with antibodies against GFP, and VP1 was detected with antibodies against TbVP1. Scale bars = 10 μm.

Figure 3. Immunoelectron microscopy of TcVtc4

A–D show different cells with gold-labeled antibodies against GFP localized in acidocalcisomes (Ac). CVC, contractile vacuole complex; G, Golgi; Mi, mitochondrion; N, nucleus; K, kinetoplast; Fl, flagellum. Scale bars in **A** and **C** = 100 nm; **B** and **D** = 500 nm.

Figure 4. Purification and polyP kinase activity of TcVtc4b

A, Bacterial lysates obtained before (U) and after induction (I) of TcVtc4b expression were analyzed by SDS-PAGE. **B**, Eluted (E1–E5) and desalted fractions obtained during TcVtc4b affinity purification as analyzed by SDS-PAGE showing a band of the expected size (53.3 kDa). **C**, Western blot analysis of lysate (Lys), flow through (FT), wash (W), eluted (E1–E6) and desalted fractions collected during TcVtc4b affinity purification, using commercial anti-histidine tag antibodies. **D**, Recombinant TcVtc4b activity as a function of ATP concentration.

Figure 5. RNA interference of *TbVtc4* in procyclic form *T. brucei*

A. In vitro growth of *T. brucei* procyclic forms is reduced by knockdown of *TbVtc4* by RNAi. *Black*, uninduced control. *Gray*, PCF cultures induced with 1 μ g tetracycline/ml. Data shown are cell densities from a representative experiment. Total cell density was calculated from all dilution factors used when passing cultures during the experiment. **B.** QRT-PCR amplification curve of cDNA produced from a representative RNAi experiment six days after induction of dsRNA. *Black*, uninduced control. *Gray*, PCF cultures induced with 1 μ g tetracycline/ml. Error bars equal 1 standard deviation of the mean from duplicate assays. The dashed line denotes the threshold used for calculation of C_t . **C.** Relative abundance of *TbVtc4* transcripts in terms of threshold cycle (C_t) normalized to the geometric mean of C_t 's for *T. brucei* ssRNA and tubulin from four different experiments. Error bars represent one standard deviation of the mean ($n = 4$).

Figure 6. Knockdown of *TbVtc4* transcripts by RNA interference increases cellular pyrophosphate (PP_i) and decreases short chain polyP levels

A. PP_i content of cells, $p < 0.05$ (ANOVA, $n = 3$). Error bars represent one standard deviation of the mean. **B.** Short chain polyP content of PCF *T. brucei* after six days of induction with $1 \mu\text{g mL}^{-1}$ tetracycline, $p < 0.05$ (Mann-Whitney test, $n = 3$, two-tailed). Error bars represent one standard deviation of the mean. **C.** Long chain polyP content of PCF *T. brucei* is unaffected by RNA interference of *TbVtc4* after six days of dsRNA induction. Error bars represent standard error of the mean.