Overexpression of a Zn$^{2+}$-sensitive Soluble Exopolyphosphatase from *Trypanosoma cruzi* Depletes Polyphosphate and Affects Osmoregulation

Received for publication, June 12, 2007, and in revised form, August 28, 2007 Published, JBC Papers in Press, September 7, 2007, DOI 10.1074/jbc.M704841200

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We report the cloning, expression, purification, and characterization of the *Trypanosoma cruzi* exopolyphosphatase (TcPPX). The product of this gene (TcPPX), has 383 amino acids and a molecular mass of 43.1 kDa. TcPPX differs from most exopolyphosphatases in its preference for short-chain polyphosphate (poly P). Heterologous expression of TcPPX in *Escherichia coli* produced a functional enzyme that had a neutral optimum pH and was dramatically inhibited by low concentrations of Zn$^{2+}$, high concentrations of basic amino acids (lysine and arginine), and heparin. TcPPX is a processive enzyme and does not hydrolyze ATP, pyrophosphate, or p-nitrophenyl phosphate, although it hydrolyzes guanosine 5'-tetraphosphate very efficiently. Overexpression of TcPPX resulted in a dramatic decrease in total short-chain poly P and partial decrease in long-chain poly P. This was accompanied by a delayed regulatory volume decrease after hyposmotic stress. These results support the role of poly P in *T. cruzi* osmoregulation.

Polyphosphate (poly P)$^8$ is a ubiquitous polymer of a few to several hundred orthophosphate residues linked by high-energy phosphoanhydride bonds. Poly P is found in the environment and in all life forms, where it is involved in a number of functions (1, 2). Poly P is essential for bacterial responses to stresses and starvation, as well as for survival and virulence (1, 2). Similar functions in adaptation to stress have been attributed to poly P in eukaryotic cells such as yeast (3, 4), fungi (5), and algae (6–8). Poly P is also involved in blood clotting (9), eukaryotic cell proliferation (10, 11), and induction of apoptosis in plasma and myeloma cells (12).

In *Trypanosoma cruzi*, the etiologic agent of Chagas disease, most poly P is accumulated in acidocalcisomes. Acidocalcisomes are acidic, calcium-containing organelles that have been demonstrated to be involved in osmoprotection (13–16). Rapid hydrolysis of acidocalcisome poly P occurs when epimastigotes of *T. cruzi* are exposed to hypotonic stress (16) resulting in increased osmolarity and swelling of the organelle (15). In addition, a microtubule and cAMP-mediated fusion of acidocalcisomes to the contractile vacuole complex with translocation of an aquaporin results in water movement and a regulatory volume decrease (15), indicating a link between acidocalcisomes and osmotic homeostasis.

In eukaryotic cells the hydrolysis of poly P is performed by the action of endopolyphosphatases and exopolyphosphatases (1, 2). Genes encoding for exopolyphosphatases from *Saccharomyces cerevisiae* (17), *Trypanosoma brucei* (18), and *Leishmania major* (19) have been cloned and expressed in *Escherichia coli*, whereas a gene encoding an endopolyphosphatase from *S. cerevisiae* (20) is the only one that has been cloned and expressed. The *L. major* exopolyphosphatase (LmPPx) has been characterized recently and demonstrated to be located in acidocalcisomes and in the cytosol (19). An exopolyphosphatase activity has also been detected in acidocalcisomes of *T. cruzi* (16). The crystal structures of bacterial (21–23) and yeast (24) exopolyphosphatases have been reported recently. In contrast to the bacterial exopolyphosphatases that belong to the sugar kinase/actin/hsp-70 superfamily, the yeast and protozoal exopolyphosphatases belong to the DHH (aspartate, histidine, and histidine) superfamily phosphoesterases to which the family II pyrophosphatases belong. Members of this superfamily contain the conserved motif DHH.

One way to investigate the role of poly P in osmoregulation in *T. cruzi* is by manipulating the expression of the genes involved in poly P metabolism and the cellular levels of poly P. Because no poly P kinase has been described in *T. cruzi*, whose gene
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knock-out could contribute to this knowledge, a feasible alternative is to alter poly P content by overexpressing PPX, which is the only enzyme described to date involved in poly P degradation in this parasite. In this work, we have overexpressed PPX in T. cruzi observing a dramatic decrease in poly P levels and higher susceptibility of these cells to osmotic stress than control cells. In addition, we have biochemically characterized the recombinant T. cruzi PPX and found it to be similar to the Leishmania major PPX (19). The results support the role of poly P in osmoregulation in eukaryotic cells.

EXPERIMENTAL PROCEDURES

Cell Culture—T. cruzi epimastigotes (Y strain) were grown at 28 °C in liver infusion tryptose medium (LIT) (25) supplemented with 5% heat-inactivated newborn calf serum. The epimastigotes transformed with pTEX constructs were maintained in liver infusion tryptose medium supplemented with 5% heat-inactivated fetal bovine serum and 0.25 mg/ml Geneticin (G418) (14). T. cruzi amastigotes and trypomastigotes (Y strain) were obtained from the culture medium of L6E9 myoblasts (14), and specific primers P3 (5'-TCCACCCTCCCA- AATCGGCA-3') and P4 (5'-ATAAACCCAGGACACCGTTTCCC-3') for 5'-RACE; and oligo(dT) primer (5'-ATGGATGCC- TACAGCGCTCTTTTTTTTTTTTTTTTTTTT-3') and specific primers P5 (5'-CAAGAAGCGGAATTGACGGT-3') and P6 (5'-AACAGCTTCTGATTCAATT-3') for 3'-RACE. PCR products were cloned into the TA-cloning PCR 2.1-TOPO vector (Invitrogen) and sequenced as above. 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 (Urbana, IL).

For T. cruzi genomic DNA library (29) screening, 3.0 × 10⁵ plaque-forming units (approximately three times the content of the library) were plated at a density of 2 × 10⁵ plaque-forming units/90-mm plate on host strain LE392. Plaques were allowed to develop to ~1.0 mm in diameter before being lifted onto nylon membranes. Membranes were probed with the [α-32P]dCTP-labeled open reading frame of TcPPX according to standard procedures. Three positive clones were isolated, and one of them was chosen for DNA sequencing through primer walking. DNA and deduced amino acid sequences were analyzed with the Wisconsin Sequence Analysis Package (version 10.0-UNIX, Genetics Computer Group, Madison, WI). The sequences of the isolated T. cruzi PPX cDNA and genomic DNA clones were deposited in the GenBankTM data base under accession numbers AF545106 and AY178275, respectively. The predicted amino acid sequence of TcPPX (AAP74699) was aligned with the sequences of other PPXs by using the Biology Workbench 3.2 utility.

Southern and Northern Blot Analysis—Total genomic DNA from epimastigotes of T. cruzi was isolated by phenol-chloroform extraction (30) digested with different restriction enzymes, separated on a 1% agarose gel, and transferred to nylon membranes. The blot was probed with [α-32P]dCTP-labeled TcPPX. After hybridization, the blot was washed three times in a × SSC, 0.1% SDS (SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 65 °C. For the Northern blot analysis, total RNA was isolated from different stages of T. cruzi using the TRIzol reagent. The polyadenylated RNA was obtained using the poly(A) tract mRNA isolation system. RNA samples were subjected to electrophoreses in 1% agarose gels containing 2 M
formaldehyde, 20 mM MOPS (pH 7.0), 1 mM EDTA, 8 mM sodium acetate, transferred to nylon membranes, and hybridized with a probe containing the entire coding sequence of TcPPX gene obtained by PCR. The TcP0 gene was used as a loading control assuming a similar level of expression of this gene in all stages (31). Densitometric analyses of Northern blots were performed by using a Kodak Digital Science Image Station 440 CF. Both TcPPX and TcP0 DNA probes were labeled with [α-32P]dCTP using random hexanucleotide primers and the Klenow fragment of DNA polymerase I (Prime-a-Gene Labeling System).

**Heterologous Expression of TcPPX in E. coli**—A PCR product was generated using primers Ex28F (5′-GCCATGGCTCGCG-TGATAATG-3′) and Ex28R (5′-GTCGACGGTATTGGCCAAGGC-3′), which included the entire ORF of TcPPX with NcoI and XhoI sites (underlined) to allow insertion in the Klenow fragment of DNA polymerase I (Prime-a-Gene Labeling System). The recombinant construct TcPPX/pET28a was transformed into E. coli BL21(DE3), and the transformants were inoculated into 1 liter of Luria-Bertani broth medium supplemented with 30 μg/ml kanamycin and grown at 37 °C. When the culture density reached an A600 of 0.5, the expression of the TcPPX gene was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested after incubation at room temperature for 6 h, and pellets were washed twice with cold PBS and kept frozen at −80 °C until use.

Bacterial cell pellets were resuspended (2.5 ml/g wet weight of pellet) in lysis/binding buffer (0.5 M NaCl, 20 mM Tris-HCl, and 5 mM imidazole, pH 7.9), incubated with 10 mg/ml lysozyme for 20 min on ice, and then sonicated 3 times at 15% amplitude for 20 s at 4 °C with 30-s intervals (Branson Sonifier 450). The lysate was incubated with 25 units of benzonase nuclease per milliliter for 20 min on ice to reduce its viscosity before being centrifuged at 20,000 × g for 30 min at 4 °C to separate the pellet and supernatant fractions. Supernatants were loaded to a His- Bind Quick 900 Cartridge and sequentially washed with 1× binding buffer and 1× washing buffer (0.5 M NaCl, 20 mM Tris-HCl, and 60 mM imidazole, pH 7.9). The recombinant TcPPX (rTcPPX) was finally eluted with 1× eluting buffer (0.5 M NaCl, 20 mM Tris-HCl, and 1 M imidazole, pH 7.9). The eluted fractions were pooled and immediately desalted in desalting buffer (50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂) using HiTrap desalting column (Amersham Biosciences). The purity of rTcPPX was determined by SDS-PAGE. Protein concentration was determined by the protein assay reagent (Bio-Rad) using bovine serum albumin as a standard.

**Antibody Generation and Purification**—The whole ORF of TcPPX amplified by the additional primer pair Ab28F (5′-GGAA-TTCTCTGCGTGATAATG-3′) and Ex28R (above) was cloned downstream of the N-terminal histidine tag of the pET28a expression vector. After the recombinant plasmid was transformed into E. coli BL21(DE3), overexpression of TcPPX was obtained through the addition of 1 mM isopropyl-β-D-thiogalactopyranoside as described above. Purification was performed under denatured conditions in which 6 M urea was included in all the buffers used above. The denatured protein solubilized from the inclusion bodies was purified using nickel-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer’s instructions. The protein purity was determined by SDS-PAGE analysis and sent to Cocalico Biologicals, Inc. (Reamstown, PA) for production of a guinea pig polyclonal antiserum. The affinity purification of anti-TcPPX serum was performed using the cyanogen bromide-activated resin from Sigma.

**SDS-PAGE and Western Blotting**—Electrophoresis was performed as described by Laemmli (32) under reducing conditions. Electrophoresed proteins were transferred to nitrocellulose membranes using a Bio-Rad transblott apparatus. Following transfer, the membrane blots were blocked with 3% fish gelatin in PBS containing 0.1% Tween 20 (PBS-T) at 4 °C overnight. Blots were first incubated with anti-TcPPX polyclonal antibody (1:3,000), or anti-α-tubulin monoclonal antibody (1:3,000), and then with horseradish peroxidase-conjugated anti-guinea pig IgG antibody (1:10,000), or goat anti-mouse antibody (1:20,000). Preimmune serum was used as control at the same concentration. Immunoblots were visualized on blue-sensitive x-ray film (Midwest Scientific, St. Louis, MO) using the ECL chemiluminescence detection kit according to the instructions of the manufacturer.

**Generation of TcPPX Expression Constructs**—The whole ORF of TcPPX was amplified by PCR using Pfu DNA polymerase and oligonucleotide primers to introduce XbaI (OvF, 5′-GGATATGATGCGCGCTTAAATG-3′) and XhoI (OvR, 5′-GTCGAGTTACAGTTTTGGCCAGAGG-3′) sites (underlined), respectively. The TcPPX PCR product was subcloned into the expression vector pTETmDigested with XbaI and XhoI restriction enzymes.

**Transfection of T. cruzi**—T. cruzi epimastigotes were grown to a density of 2 × 10⁶/ml, washed once at room temperature with electroporation buffer (137 mM NaCl, 5 mM KCl, 5.5 mM Na₂HPO₄, 0.77 mM glucose, and 21 mM Hepes, pH 7.2), and resuspended at a density of 10⁶ cells/ml in electroporation buffer. Transfections were carried out in a 4-mm gap cuvette with 100 μg of recombinant plasmid DNA extracted by Plasmid MaxiPrep kit (Qiagen). The cells were electroporated by using a Bio-Rad Gene Pulser II set at 1.5 kV and 50 microfarads with two pulses, and then incubated on ice for 5 min. Parasites were recovered in 5 ml of LIT supplemented with 5% fetal bovine serum at 28 °C, and after 24 h in culture, Geneticin was added to a final concentration of 250 μg/ml.

**Synthesis of [32P]Polyphosphate**—For extraction of poly P from E. coli, a strain overexpressing the polyphosphate kinase (NR100 P9E30 ppk*) was used. Bacterial cells were grown overnight in Luria-Bertani medium in the presence of 100 μg/ml ampicillin and 25 μg/ml kanamycin, and then inoculated to fresh medium in the presence of antibiotics and grown for ~1 h at 37 °C, or to an A₆₀₀ of 0.5. At this time, 50 μM isopropyl-β-D-thiogalactopyranoside was added to the culture to induce the overexpression of the polyphosphate kinase. After 1 h of growth, the cells were washed twice in 3-(N-morpholino)propanesulfonic acid (MOPS) minimum medium (33) containing
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10 mM K2HPO4. The culture was incubated in the same medium in the presence of antibiotics and [32P]phosphate at a final concentration of 10 mCi/ml for 2 h at 25 °C. After labeling, the cells were washed twice in the same medium without the labeled P_i, and the final pellet was resuspended in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, and 4 M guanidine isothiocyanate. Long-chain poly P was isolated according to Ault-Riché et al. (34).

Exopolyphosphatase Activity and Poly P Analysis—rTcPPX activity was assayed measuring release of P_i using the method of Lanzetta et al. (35). The standard assay mixtures contained 50 mM Tris-HCl (pH 7.5), 2.5 mM MgCl2, and 100 μM GP4 and 9.3 ng of purified rTcPPX protein. The reactions were performed in 96-well plates and started by the addition of rTcPPX. After incubation for 10 min at 30 °C, the reactions were stopped by the addition of the equal volume of the freshly prepared mixture with 3 parts of 0.045% malachite green and 1 part of 4.2% ammonium molybdate, which was filtered prior to use as described before (36). The absorbance at 660 nm was read using SpectraMax M2e plate reader ( Molecular Devices, Sunnyvale, CA) after incubating for 10 min at room temperature for color development. The amount of P_i released was determined by comparison with a standard curve. The inhibitors used (Figs. 4 and 5) did not affect the colorimetric determination of P_i (data not shown). The specific activity of rTcPPX was defined as micromoles of P_i released/min per mg of protein.

For the assays with long-chain poly P, 600 mM (in terms of P_i residues) of [32P]poly P200 (8 mCi/mmol) was added to a reaction mixture containing 250 mM sucrose, 50 mM Tris-HCl, pH 7.5, 2 mM MgCl2, and 1 μg/ml purified rTcPPX. Aliquots of 50 μl were taken at different intervals of time up to 90 min and added to a mixture of phenol:chloroform to stop the reaction. Poly P was isolated from the aqueous phase and analyzed by electrophoresis in 20% polyacrylamide/7% urea gel as described before (16).

The specific activity of rTcPPX was determined as micromoles of P_i released/min per mg of protein.

For the assays of exopolyphosphatase in control and TcPPX-overexpressing cells, the cell lysates were used immediately after preparation utilizing poly P_200 as substrate, and measuring P_i release as described above.

Extraction and Determination of Short-chain and Long-chain Poly P Levels in Epimastigotes—Cells (1 × 10^6) were harvested and washed with buffer A twice. The short-chain poly P was extracted with 0.5 M perchloric acid (HClO4), and the long-chain poly P was extracted with glass milk (Molecular Probes) as described by Ault-Riché et al. (34). The extracted poly P was determined by the amount of P_i released upon treatment with an excess of S. cerevisiae PPX1 (ScPPX1) (16). The intracellular concentration of poly P in T. cruzi epimastigotes was calculated based on the measured intracellular volume of 34.8 μl for 10^9 cells (37).

Polyphosphate Detection with DAPI Using Fluorescence Microscopy—Wild-type epimastigotes or epimastigotes overexpressing TcPPX were washed twice in PBS, pH 7.2, and fixed in 4% paraformaldehyde in PBS, pH 7.2, for 30 min at room temperature. After washing twice in PBS, pH 7.2, the fixed cells were incubated with 50 μg/ml DAPI in PBS for 15 min at room temperature and protected from light. The fluorescence was immediately observed in a Deltavision fluorescence microscope using an excitation filter of 360 nm and an emission filter >500 nm as described previously in yeast (38, 39) and trypanosomatids (16, 40). The images were recorded with a Photometrics CoolSnap HQ camera under the same exposure time and nonsaturating conditions.

RESULTS

Cloning and Sequencing of a PPX Gene from T. cruzi—The complete coding region of TcPPX was established as described under “Experimental Procedures,” and the translation of the ORF of 1152 bp yielded a polypeptide of 383 amino acid residues with a predicted molecular mass of 43.1 kDa and a pI of 5.7 (Fig. 1). A genomic DNA clone isolated by genomic library screening completely matched the corresponding cDNA sequence. A BLAST search of the protein data base showed that the amino acid sequence of TcPPX has 54%, 42, and 19% identity to T. brucei, L. major, and S. cerevisiae PPXs, respectively. The polypeptide consisted of an N-terminal DHH domain (amino acids 21–189) followed by a C-terminal DHHA2 (DHH-associate domain type 2) domain (amino acids 222–377). This domain structure is shared with the TbPPX, LmPPX, and ScPPX (Fig. 1) and a number of other eukaryotic, archaeal, and eubacterial proteins, several of which have been identified as exopolyphosphatases by sequence similarity or biochemical evidence.

Southern blot analysis was performed with the TcPPX gene as a probe. All restriction enzymes used gave single bands that were distinct from one another, suggesting the presence of a single TcPPX gene in the T. cruzi genome (data not shown).

Because the genome of the CL strain of T. cruzi has just been published (41) we searched the available genomic data for homologues of TcPPX (Y strain). A BLASTp search using the predicted TcPPX amino acid sequence as query revealed two predicted proteins (most probably corresponding to the two alleles of the gene in this hybrid strain) that differ in several amino acids from TcPPX: EAN93577.1 and EAN92516.1. The sequence of EAN93577.1 differs from TcPPX in 3 amino acids, whereas that of EAN92516.1 differs in 12 amino acids. EAN92516.1 and EAN93577.1 differ between them in 14 amino acids.

Northern Blot Analysis of TcPPX—Northern blot analysis was performed using poly(A)^+ RNA from different forms of the
parasite and the TcPPX gene as a probe. The presence of a single transcript of 1.4 kb was detected in each of the three life stages of T. cruzi. Analysis of the 1.4-kb band by densitometry indicated that the TcPPX gene was expressed at higher levels in epimastigotes than in amastigotes and trypomastigotes, whereas the transcription of a ribosomal protein gene (TcP0) was at comparable levels in all three stages of the parasite (Fig. 2A). Western blot analysis confirmed a higher expression in epimastigotes, although the protein was expressed in all stages (Fig. 2B).

Purification of Recombinant TcPPX—TcPPX was expressed in E. coli as a fusion protein with a C-terminal polyhistidine tag. Affinity chromatography on a nickel-chelated agarose column allowed protein purification. After 6 h of induction with 1 mM isopropyl-β-D-thiogalactopyranoside, the cells were disrupted, and the proteins were separated using SDS-polyacrylamide gels. Fig. 2C, lane 4, shows that the expressed protein appears as a strong band with an approximate molecular mass (43 kDa), which is very similar to the predicted molecular mass (43.1 kDa).

Reaction Requirements of the Recombinant Protein—In contrast to L. major PPX (19), TcPPX was stable in solution after purification. The purified protein could be desalted, aliquoted, and maintained at −80 °C for several months without significant loss of its activity.

The recombinant TcPPX has a high activity with poly P3 and GP as substrates (Table 1 and Fig. 3B). Very low activity could be detected with long-chain poly P (45–730 residues) by measuring phosphate release (Fig. 3A). We also investigated the reaction products formed by TcPPX acting on long-chain poly P.
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TABLE 1

| Substrates | \( V_{\text{max}}^a \) (\( \mu \text{mol/min/mg} \)) | \( K_m \) (\( \mu \text{M} \)) | \( k_{\text{cat}} \) (min\(^{-1}\)) | \( k_{\text{cat}}/K_m \) (min\(^{-1}\)\( \mu \text{M} \)) |
|------------|------------------------|-----------------|-----------------|-------------------|
| poly P\(_3\) | 31.85 ± 1.07           | 42.20 ± 5.05    | 3.46            | 8.20 \( \times 10^4 \) |
| GP\(_4\)  | 19.85 ± 0.87           | 73.39 ± 9.14    | 0.72            | 9.80 \( \times 10^3 \) |

\( ^a \) The \( K_m \) and \( V_{\text{max}} \) were calculated using SigmaPlot 10.0.

\[ ^{32}\text{P}\]poly P by 20% polyacrylamide/7% urea gel electrophoresis. Fig. 4 shows a time course of hydrolysis of \([^{32}\text{P}]\)poly P\(_{200}\). A slight Pi accumulation was detected as a result of hydrolysis of the labeled substrate (Fig. 4B). Intermediary products could not be detected. These experiments suggest that the enzyme acts as an exoenzyme in a processive mode releasing Pi residues from the ends of the chain. The enzyme is not a general phosphatase. ATP, PP\(_i\), and \( p \)-nitrophenyl phosphate were not significantly hydrolyzed (data not shown). The temperature optimum assay indicated that rTcPPX had the highest activity at 30 °C for both poly P\(_3\) and GP\(_4\) (data not shown). The pH optimum of rTcPPX was determined for poly P\(_3\) and GP\(_4\). For poly P\(_3\), rTcPPX has the maximum activity at the pH range of 7.5—8.0 (Fig. 5A), whereas for GP\(_4\), the maximum activity was at pH 7.5 (Fig. 5B).

The effect of divalent cations on the exopolyphosphatase activity is shown in Fig. 5 (C and D). The rTcPPX activity, like in other exopolyphosphatases (17, 19, 42–47), was negligible in the presence of 5 mM EDTA and was stimulated in the presence of a divalent cation as metal cofactor. At low concentrations Mg\(^{2+}\), Mn\(^{2+}\), and Co\(^{2+}\) (<1 mM) stimulated rTcPPX activity to different degrees, depending on the cation and substrate used. We also tested some compounds that have been reported to inhibit other exopolyphosphatases (17, 42, 45) on the hydrolysis of poly P\(_3\) by rTcPPX. rTcPPX was slightly inhibited by high concentrations of NaCl and KCl (Fig. 6A). Basic amino acids, such as arginine and lysine (Fig. 6B), which are abundant in acidicalcisomes (37), had a slight stimulatory effect at low concentrations (25 mM), and an inhibitory effect at high concentrations (>100 mM). Although divalent cations are a requirement for optimum activity, some of them, when tested in the presence of MgCl\(_2\), acted as strong inhibitors of this activity (Fig. 6C). CuCl\(_2\) and ZnCl\(_2\) were the most effective, inhibiting the activity at very low concentrations (Fig. 6C, inset), whereas CaCl\(_2\) showed a lower inhibitory effect even when tested at high concentrations (Fig. 6C). The recombinant enzyme was
plasmid TcPPX/pTEX was transfected in T. cruzi epimastigotes by electroporation. The transformants were selected with 250 µg/ml G418 and the total RNA was extracted for Northern blot analysis using TcPPX ORF as a probe (Fig. 7A). The transformants showed weak reactions in the wild-type epimastigotes or in the epimastigotes transfected with the empty pTEX vector when using total RNA, respectively (Fig. 7A, upper panel, lanes 1 and 2). However, a strong band corresponding to the TcPPX transcript (~1.4 kb) was detected in TcPPX/pTEX transformants (Fig. 7A, upper panel, lane 3). Western blot analysis showed that a major protein band with the correct molecular mass was present in TcPPX/pTEX transformants (Fig. 7B, upper panel, lane 4). In addition, another band representing the endogenous TcPPX (which was slightly smaller than the overexpressed protein) was also detected in all cell lines (Fig. 7B, upper panel, lanes 1–4). Overexpression of TcPPX did not cause any morphological change (data not shown) or growth defect (Fig. 8A).

Further analysis revealed that lysates of epimastigotes overexpressing TcPPX exhibited a 4.8-fold higher polyphosphatase activity than wild-type cells (Fig. 8C). The amount of long-chain poly P was only slightly decreased (Fig. 8C). The decrease in poly P content was further confirmed by DAPI staining (Fig. 8D), a technique that has been used to visualize accumulation of this polymer (16, 38–40, 49).
When epimastigotes are subjected to a 50% reduction in osmolarity (from 300 to 150 mosM) they rapidly swell and then began to shrink within a few seconds, such that after 5 min they are virtually indistinguishable in terms of motility and morphology from control cells maintained in isosmotic conditions (37). These changes were confirmed by following volume recovery over time using the light-scattering technique described previously (37), in which changes in absorbance of a cell suspension are negatively correlated with changes in cell volume (Fig. 8E). Epimastigotes in which TcPPX was overexpressed had an increased swelling and a delay in their volume recovery (Fig. 8E).

DISCUSSION

We report here that a gene, TcPPX, present in the T. cruzi genome, encodes a functional exopolyphosphatase. The ORF corresponding to TcPPX encodes a protein of 383 amino acids and a molecular mass of 43.1 kDa (Fig. 1). TcPPX belongs to the superfamily of DHH phosphoesterases (named after the conserved Asp-His-His motif). The DHH domain was originally identified as being common to the Drosophila prune protein and bacterial RecJ exonuclease (50). The additional presence of the DHHA2 (DHH-associated domain type 2) locates TcPPX to “subfamily 2” of the DHH superfamily, which includes sequences from eu- and eukaryotes. The consensus sequence for the DHHA2 domain is often found in tandem with the DHH domain, but its function is unknown. With the exception of the well-characterized exopolyphosphatases from S. cerevisiae (47, 51) and L. major (19), there has been no biochemical examination of the members of the subfamily 2 of the DHH domain superfamily.

TcPPX was overexpressed in bacteria, and the addition of a C-terminal histidine tag to the recombinant protein allowed efficient purification by affinity chromatography (Fig. 2C). The recombinant enzyme was different from the L. major and S. cerevisiae enzymes described previously. TcPPX, as LmPPX (19), had higher activity toward poly P of short-chain lengths and hydrolysis of long-chain poly P was negligible (Fig. 3A). In contrast, although the ScPPX acted on poly P of all chain lengths, a decrease in K_m of more than 4 orders of magnitude was observed when the chain length was increased from 3 up to 250 phosphate residues/molecule, and its highest specific activity was observed with chain lengths around 250 (47).

In contrast to ScPPX, whose activity is stimulated by Zn^{2+} (52, 53), but similarly to LmPPX (19), TcPPX was dramatically inhibited by very low concentrations of Zn^{2+} and millimolar concentrations of Ca^{2+} (Fig. 6C). All the exopolyphosphatases described to date are stimulated by Zn^{2+}, except for LmPPX, and the mitochondrial membrane exopolyphosphatase from S. cerevisiae that is 40% inhibited by 100 μM ZnCl_2 (52). Because vector (closed circles), and epimastigotes overexpressing TcPPX (open circles) submitted to hyposmotic stress. As controls, the same amount of isosmotic buffer was added to cells transfected with empty vector (closed triangles) or overexpressing TcPPX (open triangles). All experiments were done using the same amount of cells. Experiments were done in triplicate and the standard deviation was <5% at each time point. Error bars were omitted for clarity. Results are representative of those obtained from two independent experiments and are expressed in arbitrary absorbance units.

FIGURE 8. TcPPX overexpression in epimastigotes. A, overexpression of TcPPX did not affect cell growth, as monitored for at least 3 weeks (not shown). B, lysates from cells overexpressing TcPPX (OE) showed a 4.8-fold higher exopolyphosphatase activity than those from wild-type cells (WT). C, overexpression of TcPPX leads to a significant reduction in short-chain poly P and a slight decrease in long-chain poly P. D, DAPI staining of wild-type epimastigotes (b) or epimastigotes overexpressing TcPPX (f, d and h) show the overlay of the green (poly P) and blue (DNA) channels. a and e, are DIC images. Scale bars: 10 μm. E, regulatory volume decrease in epimastigotes. Cells suspended in iso-CI buffer were diluted with water to a final osmolarity of 150 mosM at time zero, and relative changes in cell volume were followed by monitoring absorbance at 550 nm as described under “Experimental Procedures.” Traces show epimastigotes transfected with pTEX empty expression plasmid (black line), pTEX control cells (red line), isosmotic buffer (green line), or hypotonic buffer (blue line).
the PPX activity is located in acidocalcisomes (16), it is necessary to modulate its activity. Possibly basic amino acids such as arginine and lysine (Fig. 6B), together with Zn\(^{2+}\) and Ca\(^{2+}\) (Fig. 6C), which are found in acidocalcisomes at concentrations in the millimolar range (13, 37, 54), may serve as counterions of poly P and control the action of TcPPX.

In common with both LmPPX (19) and ScPPX (45, 46, 48), TcPPX is inhibited by heparin. TcPPX is also a processive enzyme releasing P\(_i\) residues from the ends of the chain, because no intermediate products were detected upon poly P hydrolysis (Fig. 4). poly P\(_3\) hydrolysis by TcPPX could be stimulated by Co\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\) in that order (Fig. 5C), whereas all these cations were equally effective in stimulating GP\(_4\) hydrolysis (Fig. 5D). In contrast to LmPPX, TcPPX was only slightly inhibited by KCl and NaCl (Fig. 6A). The optimum pH varied according to the substrate used (Fig. 5, A and B).

Because TcPPX has an extremely low activity to hydrolyze long-chain poly P, it is possible that either an endopolyphosphatase or a different exopolyphosphatase is also present in acidocalcisomes. In this regard, endopolyphosphatases that act on long-chain poly P, generating tripolyphosphate, have been detected in several eukaryotes, including the protist *Giardia lamblia* (55), and the yeast endopolyphosphatase is localized in vacuoles (56).

Few exopolyphosphatases have been shown to preferentially hydrolyze short-chain poly P in other cells. In addition to the ScPPX1, which can cleave poly Ps of different lengths but prefers long chain poly Ps of up to 250 phosphate units (47), a 28-kDa exopolyphosphatase from *S. cerevisiae* was also shown to cleave short-chain poly P, but the *Km* value for the poly P substrates markedly increased with the decrease in the chain length of the polymer (43). The *E. coli* exopolyphosphatase (43) and the mammalian intestinal alkaline phosphatase (44) also preferentially cleave long-chain poly P. The cell envelope polyphosphatase (42) and the soluble mitochondrial exopolyphosphatase (46) from *S. cerevisiae* were also shown to hydrolyze short-chain poly P but with lower affinity than for long-chain poly P. The mechanism that enables an exopolyphosphatase to distinguish the ends of a long chain from those of a short one is intriguing (43), although multiple binding sites on distant portions of *E. coli* exopolyphosphatase were determined to be responsible for the polymer length recognition of the enzyme (57).

The involvement of poly P in the adaptation of eukaryotic cells to osmotic stress has been investigated before in yeast (3), fungi (5), and algae (6–8) following changes in the 31P NMR spectra of cells exposed to hyposmotic or hyperosmotic stresses. For example when the algae *Dunaliella salina* was submitted to hyposmotic conditions there was a rapid hydrolysis of long-chain poly P with generation of shorter poly P chains, whereas hyperosmotic stress resulted in the elongation of poly P chains (58). We have observed the same phenomenon in *T. cruzi* epimastigotes by biochemical determination of poly P content after hyposmotic and hyperosmotic stresses (16). We also found that hyposmotic stress results in a stimulation of ammonium production and its accumulation in acidocalcisomes (59). In addition, alkalization of this acidic compartment by NH\(_4\)Cl addition to the cells, or treatment with iono-

**REFERENCES**

1. Kornberg, A., Rao, N. N., and Ault-Riche, D. (1999) *Annu. Rev. Biochem.* 68, 89–125
2. Kul aw, I., and Kulakovskaya, T. (2000) *Annu. Rev. Microbiol.* 54, 709–734
3. Castro, C. D., Koretsky, A. P., and Domach, M. M. (1999) *Biotechnol. Prog.* 15, 65–73
4. Castro, C. D., Meehan, A. J., Koretsky, A. P., and Domach, M. M. (1999) *Appl. Environ. Microbiol.* 61, 4448–4453
5. Yang, Y. C., Bastos, M., and Chen, K. Y. (1993) *Biochim. Biophys. Acta* 1179, 141–147
6. Pick, U., Zeelon, O., and Weiss, M. (1991) *Plant Physiol.* 97, 1226–1233
7. Pick, U., and Weiss, M. (1991) *Plant Physiol.* 97, 1234–1240
8. Weiss, M., Dental, M., and Pick, U. (1991) *Plant Physiol.* 97, 1241–1248
9. Smith, S. A., Mutch, N. J., Baskar, D., Rohloff, P., Docampo, R., and Morrissey, J. H. (2006) *Proc. Natl. Acad. Sci. U. S. A.* 103, 903–908
10. Wang, L., Fraley, C. D., Faridi, J., Kornberg, A., and Roth, R. A. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 11249–11254
11. Shiba, T., Nishimura, D., Kawazoe, Y., Onodera, Y., Tsutsumi, K., Nakamura, R., and Ohshiro, M. (2003) *J. Biol. Chem.* 278, 26788–26792
12. Hernandez-Ruiz, L., Gonzalez-Garcia, I., Castro, C., Brieva, J. A., and Ruiz, F. A. (2006) *Haematologica* 91, 1180–1186
13. Docampo, R., de Souza, W., Miranda, K., Rohloff, P., and Moreno, S. N. (2005) *Nat. Rev. Microbiol.* 3, 251–261
14. Montalvetti, A., Rohloff, P., and Docampo, R. (2004) *J. Biol. Chem.* 279, 38673–38682
15. Rohloff, P., Montalvetti, A., and Docampo, R. (2004) *J. Biol. Chem.* 279, 52270–52281
16. Ruiz, F. A., Rodrigues, C. O., and Docampo, R. (2001) *J. Biol. Chem.* 276, 26114–26121
17. Wurst, H., Shiba, T., and Kornberg, A. (1995) *J. Bacteriol.* 177, 898–906
18. Lemercier, G., Bakalar, N., and Santarelli, X. (2003) *J. Chromatogr. B. Analit. Technol. Biomed. Life Sci.* 786, 305–309
19. Rodrigues, C. O., Ruiz, F. A., Vieira, M., Hill, J. E., and Docampo, R. (2002) *J. Biol. Chem.* 277, 50899–50906
20. Sethuraman, A., Rao, N. N., and Kornberg, A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 8542–8547
21. Rangarajan, E. S., Nadeau, G., Li, Y., Wagner, J., Hung, M. N., Schrag, J. D., Cylger, M., and Matte, A. (2006) *J. Mol. Biol.* 359, 1249–1260
22. Alvarado, J., Ghosh, A., Janovitz, T., Jauregui, A., Hasson, M. S., and Sand-
