Published in final edited form as:

Mol Microbiol. 2013 November ; 90(4): . doi:10.1111/mmi.12392.

Evidence for the Role of Vacuolar Soluble Pyrophosphatase and Inorganic Polyphosphate in *Trypanosoma cruzi* Persistence*

Melina Galizzi¹, Juan M. Bustamante¹, Jianmin Fang¹, Kildare Miranda^{1,2}, Lia C. Soares Medeiros², Rick L. Tarleton¹, and Roberto Docampo^{1,#}

¹Center for Tropical and Emerging Global Diseases and Department of Cellular Biology, University of Georgia, Athens, GA 30602

²Instituto de Biofísica Carlos Chagas Filho, Federal University of Rio de Janeiro, Cidade Universitária, Bloco G, Rio de Janeiro, Brazil

Summary

Trypanosoma cruzi infection leads to development of a chronic disease but the mechanisms that the parasite utilizes to establish a persistent infection despite activation of a potent immune response by the host are currently unknown. Unusual characteristics of *T. cruzi* are that it possesses cellular levels of pyrophosphate (PP_i) at least ten times higher than those of ATP and molar levels of inorganic polyphosphate (polyP) within acidocalcisomes. We characterized an inorganic soluble EF-hand containing pyrophosphatase from *T. cruzi* (TcVSP) that, depending on the pH and cofactors, can hydrolyze either pyrophosphate (PP_i) or polyphosphate (polyP). The enzyme is localized to both acidocalcisomes and cytosol. Overexpression of *TcVSP* (*TcVSP-OE*) resulted in a significant decrease in cytosolic PP_i, and short and long chain polyP levels. Additionally, the *TcVSP-OE* parasites showed a significant growth defect in fibroblasts, less responsiveness to hyperosmotic stress, and reduced persistence in tissues of mice, suggesting that PP_i and polyP are essential for the parasite to resist the stressful conditions in the host and to maintain a persistent infection.

Introduction

An unusual characteristic of *Trypanosoma cruzi*, the etiologic agent of Chagas disease, is that it possesses cellular levels of pyrophosphate (PP_i) that are at least ten times higher than those of ATP (Urbina *et al.*, 1999). In addition, they store PP_i and polyphosphate (polyP), which is a polymer of orthophosphate residues linked by high-energy phosphoanhydride bonds, in acidocalcisomes (Docampo *et al.*, 1995). Acidocalcisomes from *T. cruzi* are characterized by their high electron density, high content of cations bound to PP_i and polyP, and a number of pumps in their membranes, among them a vacuolar proton pyrophosphatase (V-H⁺-PPase or VP1), which is important for their acidification. PolyP is present in acidocalcisomes at molar concentrations (as expressed in orthophosphate units). Previous work has shown that acidocalcisomes and polyP are important for protection of the parasites against osmotic challenges similar to those present in the host (Li *et al.*, 2011). For example, bloodstream trypomastigotes must be able to resist up to 1,400 mosmol l⁻¹ when passing through the renal medulla and return to the isosmotic environment of the general circulation (Lang, 2007). In addition to its use by the acidocalcisome V-H⁺-PPase (Scott *et al.*, 1998),

*To whom correspondence should be addressed: Center for Tropical and Emerging Global Disease and Department of Cellular Biology, 350B Paul D. Coverdell Center, University of Georgia, Athens, GA 30602. Tel.: 706-542-8104; Fax: 706-542-9493; rdocampo@uga.edu.

T. cruzi PP_i can also be used as an energy donor in the reaction catalyzed by phosphate pyruvate dikinase, which is localized to the glycosomes (Bringaude *et al.*, 1998).

Inorganic pyrophosphatases (PPases) catalyze the hydrolysis of PP_i to orthophosphate (P_i). These are essential enzymes, providing a thermodynamic pull for biosynthetic reactions (Kornberg, 1962). Inorganic PPases include membrane-bound H⁺-, Na⁺- and H⁺/Na⁺-pumping PPases, and soluble PPases. The H⁺, Na⁺ and H⁺/Na⁺-PPases utilize the energy released by hydrolysis of PP_i to transport protons (Scott *et al.*, 1998; Baltscheffsky *et al.*, 1999; Maeshima, 2000) or sodium (Malinen *et al.*, 2007; Malinen *et al.*, 2008; Luoto *et al.*, 2013) across the membrane of cells or organelles. Three families of non-homologous soluble inorganic PPases have been described: family I PPases, which are present in all types of organisms and prefer Mg²⁺ as cofactor (Tammenkoski *et al.*, 2005; Tammenkoski *et al.*, 2007), family II PPases, which are exclusive to bacteria and prefer Mn²⁺ as cofactor (Young *et al.*, 1998; Tammenkoski *et al.*, 2005; Tammenkoski *et al.*, 2007), and family III, which are modified haloalkane dehalogenases present in a few bacterial species (Lee *et al.*, 2009). Several family I recombinant PPases, such as those of *Saccharomyces cerevisiae* (Oksanen *et al.*, 2007), *Trypanosoma brucei* (Lemerrier *et al.*, 2004) and *Leishmania amazonensis* (Espiau *et al.*, 2006) display activity on polyP in the presence of transition metal ions such as Zn²⁺, Mn²⁺ and Co²⁺, some of which (Zn²⁺) are abundant in acidocalcisomes (Docampo and Moreno, 2011).

T. cruzi, as *T. brucei* (Lemerrier *et al.*, 2004), and *L. amazonensis* (Espiau *et al.*, 2006), possesses two family I soluble PPases. One of these PPases is characterized by the presence of an EF-hand domain and has been termed vacuolar or acidocalcisomal soluble PPase (VSP) (Lemerrier *et al.*, 2004). This is because the enzymes from *T. brucei* (Lemerrier *et al.*, 2004) and *L. amazonensis* (Espiau *et al.*, 2006) are predominantly located in acidocalcisomes, although no evidence for this localization in *T. cruzi* has yet been presented.

Inorganic polyP has been proposed as playing a major regulatory role in the transition to bacterial dormancy and in survival under various growth-limiting conditions (Rao *et al.*, 1999), but little is known of its role in eukaryotic microbes. In this work we characterized biochemically the *T. cruzi* EF-hand domain-containing pyrophosphatase (TcVSP). By overexpressing this enzyme in *T. cruzi* we were able to increase the PPase activity up to 7-fold compared to the wild type cells. As expected, these cells showed a decrease in cytosolic PP_i, and in short and long chain polyP levels. Trypomastigotes overexpressing this enzyme were less responsive to hyperosmotic stress and less infective in tissue culture fibroblasts. In addition, the *TcVSP* overexpressing parasites (*TcVSP-OE*) were compromised for growth *in vivo*, failing to maintain persistent infection in mice. These results support roles for PP_i and/or polyP in resistance to the stressful conditions in the host and in maintenance of persistent infections that lead to Chagas disease.

Results

Cloning and characterization of TcVSP

The complete open reading frame of the putative *TcVSP* yielded a polypeptide of 414 amino acid residues with a predicted molecular mass of 47.9 kDa and a pI of 5.95. A BLASTP analysis of GenBank database showed that the amino acid sequence of the putative TcVSP is 68% and 61% identical to *T. brucei* and *L. amazonensis* vacuolar soluble pyrophosphatases (VSPs), respectively. Similar to other VSPs (Lemerrier *et al.*, 2004; Espiau *et al.*, 2006), the putative TcVSP possesses the 13 essential and active site residues conserved in all Family I soluble pyrophosphatases (Fig. S1A, *arrowheads*). One potential calcium-EF-hand binding domain was found near the *N*-terminus, as occurs with the *L.*

amazonensis and *T. brucei* enzymes (Fig. S1A, *shaded in grey*). This domain has been shown to be important for oligomerization of the protein (Lemercier *et al.*, 2004). The sequence is 100% identical to TcCLB.53503613.60 and 99% identical to TcCLB.53511165.40 available in TritypDB.org, which probably correspond to different alleles of the same gene.

Southern blot analysis was performed using the extracted genomic DNA from epimastigotes, linearized with restriction enzymes. The blot was hybridized with *TcVSP* gene probe (Fig. S1B). The presence of a single reaction band in all lanes (Fig. S1B) suggested the presence of *TcVSP* as a single copy gene in the Y strain, which is in agreement with the genome information reported for the CL strain. The expression of *TcVSP* in the three stages was investigated by western blot analysis. Several bands (see below) close to the predicted apparent molecular weight of 47.9 kDa were detected (Fig. S1C, and see below). *TcVSP* is expressed in all stages and epimastigotes and culture-derived trypomastigotes showed higher expression (Fig. S1C).

Activity of recombinant TcVSP

TcVSP was expressed in *E. coli* as a fusion protein with a C-terminal polyhistidine tag. In Fig. S1D, *lane rTcVSP* shows that the bacterially expressed protein (including the his-tag) appears as a strong single band with an approximate molecular mass (45 kDa), which is slightly lower than the predicted molecular mass (47.9 kDa).

rTcVSP, like other PPases, required the presence of a divalent cation for activity (Fig. 1A). MgCl₂ was the preferred cofactor for PP_i hydrolysis (Fig. 1A). The highest stimulation was observed at Mg²⁺ concentration of ~0.5 mM (Fig. 1A). The rTcVSP was also able to hydrolyze polyP₃, polyP₇₅ and guanosine 5'-tetraphosphate (GP₄), especially when Co²⁺ or Zn²⁺ were used as cofactors (Fig. 1B-D), although the specific activity in these cases was much lower than with PP_i (Figs. 1 and S2). Table 1 shows the enzyme has lower K_m and much higher k_{cat}/K_m with PP_i as substrate.

The pH optimum was investigated using PP_i, polyP₃, polyP₇₅, and GP₄ as substrates (Fig. 1E-H). For PP_i, rTcVSP had the maximum activity at pH 8.5 (Fig. 1E); whereas for polyP₃, polyP₇₅, and GP₄, rTcVSP had a relatively narrow pH range (pH 6.0-7.0) with the highest activity at pH 6.5 (Figs. 1F-H). The optimum temperature, with PP_i substrate, was 30°C (data not shown).

Localization of TcVSP

Previous studies had shown that *L. major* and *T. brucei* orthologues of TcVSP mainly localize to acidocalcisomes and this was the reason for their denomination as vacuolar soluble pyrophosphatases. We used several methods to define the localization of TcVSP. A TcVSP-GFP fusion expression construct was generated to transfect epimastigotes. Stably growing transformants were selected with G418 for several weeks. Transformants expressed TcVSP-GFP, which had a predominantly cytosolic localization (Fig. 2A-B), similar to that of transformants expressing GFP alone (Fig. 2C-D). We confirmed C-terminal tagging of TcVSP by western blot analysis using antibodies against GFP (Fig. 2G). Western blot analysis using antibodies against TcVSP was also done to confirm labeling of endogenous and overexpressed TcVSP with and without GFP (Fig. 2H).

Although purified antibody raised against TcVSP performed well in western blot analysis (Fig. S1C), it produced a punctate signal when used in immunofluorescence assays (Fig. 2E-F). However, affinity purified antibody did produce satisfactory signals by immunogold electron microscopy (Fig. 3). Figs. 3 shows that TcVSP (*arrowheads*) co-localizes with TcVP1 (*arrows*), the acidocalcisome marker. In addition, gold particles corresponding to the

reaction of TcVSP antibodies were also detected in the cytosol of the parasites (Fig. 3A, *upper left panel, arrowhead*). To further confirm the intracellular location of TcVSP, we performed subcellular fractionation of wild type and epimastigotes transfected with GFP/pTREX expression plasmid as a cytosolic control. Western blot analysis with anti-TcVSP antibody showed predominant reaction in the soluble fraction and some weaker reaction in the pellet fraction, while cytosolic GFP appeared predominantly in the soluble fraction and TcVP1, a marker for acidocalcisomes, only appeared in the pellet fraction (Fig. 3B). This correlated well with the subcellular localization of the aminomethyldiphosphonate (AMDP)-sensitive (TcVP1) and AMDP-insensitive (TcVSP) pyrophosphatase activity of the different fractions (Fig. 3C). It is possible that part of the TcVSP signal in the soluble fraction (Fig. 3B) may come from TcVSP present in acidocalcisomes that are disrupted during fractionation and subsequent steps for western blot analysis. Larger amounts of intact acidocalcisomes could be present for the activity experiments (Fig. 3C) as the procedure for its determination does not require additional steps. Taken together, these results are in agreement with a cytosolic and acidocalcisome localization of TcVSP, and its soluble nature.

Post-translational modifications of TcVSP

While SDS-PAGE analysis of rTcVSP resulted in the detection of only one band of apparent molecular mass of 45 kDa (Fig. S1D), western blot analyses of endogenous and overexpressed protein led to the identification of several bands (Figs. S1C, 2G, 2H). Figs. S3A and S3B, show the identification of 3-4 bands under optimal conditions. To assess the identities of the different bands, SDS-PAGE was done and these bands were excised and analyzed by mass spectrometry. Table S1 shows that the peptides in these bands identified above a 1% false discovery rate correspond only to TcVSP.

Lysates were also analyzed to determine whether post-translational modifications could be responsible for the appearance of multiple bands in western blots. As shown in Fig. S3B, the protein was sensitive to Lambda protein phosphatase, a Mn^{2+} dependent protein phosphatase with activity for phosphorylated threonine, serine and tyrosine residues. Similar experiments using PNGase F treatment, which cleaves between the innermost N-acetylglucosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins, also altered the pattern of bands (Fig. S3C). These results suggest that phosphorylation and N-glycosylation are among the post-translational modifications of this protein.

Overexpression of TcVSP in *T. cruzi*

Western blot analysis showed that protein bands with the correct molecular mass were present in the *TcVSP*/pTREX transformants (Fig. 4A, *lane 2*), while weaker bands were seen in wild type cells (Fig. 4A, *lane 1*), and a band of similar size was detected in the rTcVSP preparation (Fig. 4A, *lane 3*). Lysates of epimastigotes overexpressing *TcVSP* exhibited a 7-fold higher pyrophosphatase activity (measured with PP_i) than wild-type cells or cells transfected with GFP alone (Fig. 4B). Overexpression of *TcVSP* (*TcVSP-OE*) in epimastigotes did not cause any evident morphological change in these cells and did not affect their replication (Fig. S3D). However, when differentiated metacyclic trypomastigotes were used to infect fibroblasts, the replication of *TcVSP-OE* amastigotes was at much lower rate than that of wild-type cells (Figs. 4C and 4D).

PP_i and polyP Levels in TcVSP-OE epimastigotes

Overexpression of *TcVSP* in epimastigotes led to a significant reduction in the levels of short-chain polyP (about 20%) (Fig. 5A), and total PP_i (about 30%) (Fig. 5C), a considerable decrease in long-chain polyP (6-fold) (Figs. 5B), and no change in intracellular

ATP levels (Fig. 5F). Since most PP_i is localized in acidocalcisomes (Docampo et al., 2011b), we investigated whether cytosolic or organellar PP_i was affected in cells overexpressing *TcVSP*. The distribution of PP_i in cytosolic and organellar fractions of epimastigotes was determined by lysis of the plasma membrane by freeze-thaw cycling in a hypotonic buffer. Wild type cells had more than five times the concentration of PP_i in the soluble fraction than OE mutants (Fig. 5D). The efficiency of this fractionation was verified by measuring pyruvate kinase activity in both the supernatant (i.e. cytosolic) and the pellet (i.e. organellar) fractions. Over 90% of the measured pyruvate kinase activity was found in the cytosolic fraction (Fig. S3E). These results demonstrate effective lysis of the plasma membrane without significant disruption of organelles containing PP_i (i.e. acidocalcisomes) and indicate that the total cellular differences in PP_i (Fig. 5C) are mainly the result of different concentrations of PP_i in the cytosol (Fig. 5D), which is in agreement with the predominant cellular localization of TcVSP (Fig. 3).

Transmission electron microscopy of intact cells (Fig. 6) revealed that although the number of acidocalcisomes did not change significantly upon overexpression of *TcVSP*, their diameter, and area increased significantly, doubling their volume (Fig. 6B, and Table 2).

Response of TcVSP-OE trypomastigotes to hyperosmotic stress

Previous work has shown that polyP has a critical role in survival of trypanosomes under osmotic stress (Ruiz *et al.*, 2001; Docampo *et al.*, 2011a; Li *et al.*, 2011). Bloodstream trypomastigotes are exposed to hyperosmotic stress when circulating through the renal medulla of the mammalian host and an inadequate response to this stress could result in damaging effects. The potential effect of *TcVSP* overexpression in resistance to hyperosmotic stress was evaluated. Shrinkage of *TcVSP-OE* trypomastigotes under hyperosmotic stress was significantly reduced as compared to that of wild type cells (Fig. 7).

Virulence in mice of TcVSP-OE trypomastigotes

To investigate the relevance of the marked decrease in PP_i and polyP in *TcVSP-OE* parasites on mammalian infectivity, we infected immunocompetent C57BL/6 mice with wild type and *TcVSP-OE* trypomastigotes. Mice infected with either wild type or *TcVSP-OE* parasites survived acute infection. However, in contrast to infection with wild type *T. cruzi*, *TcVSP-OE* infected mice did not exhibit detectable parasitemias throughout the infection (Fig. 8A). Immunosuppression with cyclophosphamide (at ~150 dpi) (Bustamante *et al.*, 2008; Canavaci *et al.*, 2010) revealed persistent infection in mice infected with wild type but not *TcVSP-OE T. cruzi* (Fig. 8A). Hemocultures from *TcVSP-OE* infected and immunosuppressed mice yielded only 1 out of 8 mice positive and parasite DNA was undetectable in heart and in the majority of skeletal muscle, kidney and liver samples from *TcVSP-OE* infected and immunosuppressed mice (Fig. 8B-D and not shown). Similar outcome of undetectable parasites in blood after immunosuppression were found in Balb/c mice infected with *TcVSP-OE* parasites (Fig. S4A). Balb/c mice infected with wild type *T. cruzi* parasites showed high mortality rate (80%) after infection while all the *TcVSP-OE* infected mice remained alive throughout the entire study (Fig. S4B).

To further document the decreased virulence of the *TcVSP-OE* parasites, we infected highly susceptible C57BL/6 interferon- (IFN-)-knockout mice with wild type and *TcVSP-OE* parasites and compared their parasitemias, survival, histology and DNA detection in tissues. Although few of the mice inoculated with *TcVSP-OE* parasites had detectable parasites in the blood and all survived past that of mice infected with wild-type parasites (Fig. 9A-B), all *TcVSP-OE*-infected mice had to be euthanized between 35 and 48 dpi due to severe back limb paralysis. PCR provided evidence of parasite persistence in these animals (Fig. 9C-D and data not shown).

Collectively, these results indicate that *TcVSP-OE* parasites are less virulent and capable of being cleared from immunocompetent hosts. In all cases where *TcVSP-OE* parasites were recovered from animals, western blot analyses showed that they maintained the overexpression of *TcVSP* (see for example Fig. S4C and D). Epimastigotes obtained from these hemocultures also showed lower levels of PP_i and polyP (Fig. S4E and S4F).

Generation of protective *T. cruzi* specific CD8⁺ T cells responses upon infection with *TcVSP-OE* parasites

Low virulence strains of *T. cruzi* that induce potent immune responses but which do not persist indefinitely may have utility as live vaccines (Collins et al., 2011; Martin et al., 2006). To explore whether C57BL/6J mice infected with *TcVSP-OE* parasites elicit a detectable *T. cruzi*-specific immune response, the frequency of CD8⁺ TSKB20-specific T cells (specific for the immunodominant TSKB20 epitope encoded by genes belonging to the *trans*-sialidase (TS) family (Martin et al., 2006)) was determined. As expected based upon lower parasite load in the *TcVSP-OE*-infected mice (Martin et al., 2006; Bustamante et al., 2008), the frequency of antigen experienced (CD44^{hi}) TSKB20-specific CD8⁺ T cells in *TcVSP-OE* infected mice was lower than that displayed by their wild type infected counterparts (Fig. 10A). In addition, *T. cruzi*-specific CD8⁺ T cells in *TcVSP-OE* infected mice showed an upregulation of CD127 and lower expression of KLRG1, markers of memory maintenance and repetitive antigen stimulation, respectively, as compared with the *T. cruzi*-specific CD8⁺ T cells in wild type infected mice (Fig. 10B-C).

Based on the relatively strong *T. cruzi*-specific adaptive immune responses elicited by the host upon infection with *TcVSP-OE* parasites, we next asked if these responses were sufficient to protect mice against a virulent re-challenge with *T. cruzi*. To address this question *TcVSP-OE* infected mice and naïve mice were challenged in each footpad with 2.5×10^5 CL trypomastigotes expressing the TdTomato fluorescent protein, and parasite load at the site of infection was monitored after challenge using *in vivo* whole imaging technology as previously described (Canavaci et al., 2010; Collins et al., 2011). *TcVSP-OE* infected mice showed a better control of parasites at the site of infection compared to wild type infected mice, as indicated by the decrease in the fluorescence intensity, and therefore in the number of parasites, determined in the footpads of these animals (Fig. 10D). Collectively these results suggest that infection with the attenuated *TcVSP-OE* parasites can elicit a strong *T. cruzi* specific adaptive immune response capable of protecting mice upon a virulent secondary challenge with *T. cruzi*.

Discussion

We report here that a gene, *TcVSP*, present in the *T. cruzi* genome, encodes a functional pyrophosphatase. The gene is single copy and is expressed in all stages of *T. cruzi*. The encoded protein is soluble and localizes predominantly to the cytosol and also to acidocalcisomes of the parasites. The recombinant enzyme is similar to *T. brucei* VSP (Lemercier et al., 2004), and *L. amazonensis* VSP (Espiau et al., 2006), with respect to its divalent cation requirement, optimum pH, and ability to hydrolyze polyP at weakly acidic pH in the presence of transition metals. This latter activity is much lower, compared to its pyrophosphatase activity. The results suggest that the enzyme is, in addition, post-translationally modified by phosphorylation and *N*-glycosylation.

Overexpression of *TcVSP* in epimastigotes resulted in a 3-fold decrease in their cytosolic PP_i content and a 6-fold decrease in their amount of long chain polyP. This decrease correlated with an increase in the diameter, volume and area occupied by acidocalcisomes in *TcVSP-OE* epimastigotes, suggesting water influx to compensate the increase in osmolytes (orthophosphate) resulting from hydrolysis of polyP, as it has been reported before during

hyposmotic stress (Rohloff *et al.*, 2004). No significant changes in intracellular ATP, or growth in axenic cultures were detected in these cells. The lack of changes in ATP levels is in agreement with the idea that polyP is not an efficient supply of energy because the metabolic turnover of ATP is considerably higher than that of polyP (Rao *et al.*, 1999; Docampo *et al.*, 2005). However, when differentiated into trypomastigotes, the parasites overexpressing the enzyme were less responsive to osmotic changes and less virulent in tissue culture fibroblasts, and in mice.

T. cruzi stores polyP at remarkably high levels. Two classes of polyP are present in trypanosomes and are arbitrarily divided into short chain (from 3 to ~300 phosphate units) and long chain (from ~300 to ~1,000 phosphate units) (Moreno and Docampo, 2013) and their levels reach about 50 mM and 3 mM, respectively, in epimastigotes, and 25 mM and 0.1 mM, respectively, in amastigotes, assuming distribution across the entire volume of the cells, or molar levels within acidocalcisomes, where they accumulate (Docampo *et al.*, 2005). PolyP is a polyanion and it binds to cations such as calcium, magnesium, sodium, and potassium, as well as to basic amino acids. This binding reduces the availability of free ions in the cell and helps control the ionic strength of the cytosol. The relevance of this process is underscored by experiments showing that rapid hydrolysis or synthesis of acidocalcisome polyP in *T. cruzi* occurs during hypo- or hyperosmotic stress, respectively (Ruiz *et al.*, 2001). Osmoregulation is very important for *T. cruzi* as the parasite is submitted to dramatic changes in osmolarity during its life cycle. While traveling through the kidney, the trypomastigote needs to resist 1,300-1,400 mosmol l⁻¹ (Lang, 2007), and while in the feces and urine of the insect vector, 1,000 mosmol l⁻¹ (Kollien *et al.*, 2001). Some organs that are infected by *T. cruzi* (liver, spleen, lymphoid tissues) have also higher osmolarity than serum (330 vs. 300 mosmol l⁻¹) (Go *et al.*, 2004). In addition, all cells need to regulate their volume continuously (Lang, 2007). Under hyposmotic conditions there is an increase in ammonium production and alkalization of acidocalcisomes (Rohloff *et al.*, 2006). This leads to polyP hydrolysis (because polyphosphatases are less active at the very low pH of acidocalcisomes), increase in the number of free osmolytes (as cations are bound to the polyP polymer), and swelling of the organelles (Rohloff *et al.*, 2004). Conversely, under hyperosmotic conditions, there is an initial increase in polyP synthesis, which would result in cation uptake by acidocalcisomes and a decrease in the ionic strength of the cytosol, which otherwise would be toxic to the cells (Li *et al.*, 2011). After several hours there is a decrease in polyP, and the resulting release of cations helps restore the cytosolic ionic strength when water is taken up (Li *et al.*, 2011). Overexpression of an enzyme that cleaves polyP (exopolyphosphatase, TcPPX) (Fang *et al.*, 2007b) was also shown to decrease polyP and affect osmoregulation, and we now report that overexpression of another polyphosphatase (TcVSP) is important not only for osmoregulation but also for the establishment of infection *in vitro* and *in vivo*.

PolyP function has been studied mainly in bacteria but little is known of its functions in eukaryotic cells. PolyP, which in bacteria is mainly of long-chain type (up to 1,000 phosphate units), has been reported to be important for virulence of different bacteria, such as *Salmonella* spp., *Shigella flexneri*, *Vibrio cholerae*, *Neisseria meningitides*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* but the mechanism involved is not known (Brown *et al.*, 2008). A reduction in polyP content in mycobacteria has been associated with defective survival in macrophages and reduced persistence in animals (Sureka *et al.*, 2007).

The fact that *TcVSP* overexpressors fail to establish a persistent infection in mice suggests that either the increased osmotic fragility of the parasites as a result of changes in PP_i and polyP levels impacts the growth and survival of the parasites or that these *TcVSP-OE* parasites are more susceptible to phagocytic control. The large amount of polyP and PP_i in trypanosomes (Ruiz *et al.*, 2001), which is probably released upon their phagocytosis also

suggest another mechanism. It has recently been proposed (Salmon et al., 2012) that upon phagocytosis of *T. brucei* by myeloid cells, activation of the parasite adenylyl cyclase leads to the increase in cyclic AMP (and PP_i), and activation of a protein kinase A that stimulates TNF- synthesis and increases virulence, suggesting that parasite molecules could have a role in modulation of infection at the host-parasite interface.

In conclusion, overexpression of *TcVSP* produces parasites that have lower levels of PP_i and polyP and therefore a defective response to osmotic stress as well as defective growth *in vitro*. Infected mice are able to clear the infection and parasites are unable to persist in tissues. These parasites elicit a strong *T. cruzi*-specific adaptive immune response capable of protecting mice upon a virulent secondary challenge with the parasites. Our results also suggest an important regulatory role of PP_i and polyP in infection by *T. cruzi*.

Experimental procedures

Chemicals and reagents

Fetal bovine serum was from Atlanta Biologicals. Dulbecco's phosphate buffer saline (PBS), protease inhibitor cocktails (P8849 and P8340), paraformaldehyde, glutaraldehyde, bovine serum albumin, malachite green, ATP, PP_i, polyphosphate glass (polyP₇₅), guanosine 5'-tetrakisphosphate (GP₄), and monoclonal antibody against tubulin were purchased from Sigma (St. Louis, MO). Tripolyphosphate (polyP₃) was from Fluka. Restriction enzymes were from New England BioLabs (Ipswich, MA), T4 DNA ligase, *Taq* polymerase, DNA ladder, and goat serum were from Gibco BRL, Life Technologies, Inc. (Gaithersburg, MD). The pET28a⁺ expression system, nickel-nitriloacetic acid His-Bind resin, and benzonase nuclease were from Novagen Inc. (Madison, WI). pCR2.1-TOPO cloning kit was from Invitrogen (Carlsbad, CA). Hybond-N nylon membrane, and ECLTM chemiluminescence kit were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). [γ-³²P]dCTP (3.0 Ci/mol) was from Perkin Elmer. Coomassie Brilliant Blue protein assay reagent was from Bio-Rad. Peptide:N-glycosidase F (PNGase F; Cat # P0704S) and Lambda protein phosphatase (Cat # P0753S) were from New England Biolabs, Inc (Ipswich, MA). Antibodies against TbVP1 were a gift from Norbert Bakalara (University of Montpellier, France). Antibodies against GFP were from Invitrogen (Life Technologies, Grand Island, NY). All other reagents were analytical grade.

Cell Culture

T. cruzi epimastigotes (Y strain) were grown at 28°C in liver infusion tryptose medium (LIT) (Bone et al., 1956) supplemented with 5% heat-inactivated newborn calf serum. The epimastigotes transformed with pTREX constructs were maintained in LIT medium supplemented with 5% heat-inactivated fetal bovine serum and 0.25 mg/ml geneticin (G418) (Montalvetti et al., 2004). *T. cruzi* wild type amastigotes and trypomastigotes (Y or CL strains) were obtained from the culture medium of L₆E₉ myoblasts using a modification of the method of Schmatz and Murray (Schatz et al., 1982) as described previously (Moreno et al., 1994). L₈E₆₄ myoblasts, used to maintain *TcVSP*-overexpressing cells, were from American Type Culture Collection. Vero cells and human foreskin fibroblasts were also used where indicated.

In vitro infection assays

Human foreskin fibroblasts (HFF) were seeded on glass coverslips and allowed to grow into a semi-confluent monolayer in media (DMEM Low Glucose, Sigma#D-5523) supplemented with 10% Cosmic calf serum from Atlanta Biologicals and 0.1% L-glutamine from Hyclone) and incubated overnight at 35°C, 7% CO₂. Trypomastigotes were then added at a 1:10 multiplicity of infection (6 × 10⁶ trypomastigotes/coverslip). At 2 h postinfection, coverslips

were washed 3 times with Hanks solution to remove any extracellular parasites and fresh medium was added. The coverslips were then incubated at 35°C, 7% CO₂ for 53 h. After incubation, coverslips were washed once with PBS pH 7.4 and fixed with 2% paraformaldehyde in PBS for 1 h at room temperature. After fixation, coverslips were gently washed 5 times with PBS, pH 7.4, and mounted in glass slides using Fluoromount-G (from Southern Biotech) containing 15 µg/ml of DAPI (to stain host and amastigotes DNA). The quantification of the infection was done using an Olympus IX71 microscope with an eyepiece counting grid. Microscopy fields were randomly selected for each coverslip. For each selected microscopy field the total number of host cells, total number of infected cells (cells that had at least one intracellular amastigote) and the number of amastigotes per infected cell were recorded. A total of at least 100 cells were quantified per independent experiment.

Southern blot analysis

The genomic DNA from epimastigotes (Y strain) of *T. cruzi* was isolated by phenolchloroform extraction (Sambrook, 1989), digested with different restriction enzymes (ClaI, HindIII, NcoI, SalI and XbaI), separated on a 0.7% agarose gel, and transferred to nylon membranes. The blot was probed with [³²P]dCTP-labeled *TcVSP* using the Klenow fragment of DNA polymerase I (Prime-a-Gene Labeling System, Promega). After hybridization, the blot was washed three times in a 1 × SSC, 0.1% SDS (1×SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at 65 °C. The blot was exposed on blue-sensitive x-ray film (Midwest Scientific, St. Louis, MO) at –80 °C for 16 h.

Transfection of *T. cruzi*

T. cruzi epimastigotes were grown to a density of 2 × 10⁷/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 10⁸ cells/ml in electroporation buffer. Transfections were carried out in a 4-mm gap cuvette with 100 µg recombinant plasmid DNA. 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 (G418) was added to a final concentration of 250 µg/ml to select stably growing transformants.

Generation of *TcVSP* overexpression constructs

The entire open reading frame of *TcVSP* was amplified by PCR using *Pfu* DNA polymerase and oligonucleotide primers to introduce XbaI (5' - GTCTAGATGTCTGAAAATAAGGCGAC-3') and XhoI (5' - GCTCGAGTTACACCTCTTCCTTCTTATTTATC-3') restriction sites (underlined), respectively. The *TcVSP* PCR product was cloned downstream of the ribosomal promoter in the expression vector pTREX, which was linearized using XbaI and XhoI restriction enzymes to excise the inserted DNA fragment of yellow fluorescence protein. Primers 5' - GTCTAGATGTCTGAAAATAAGGCGACG-3' and 5' - GGAATTCCACCTCTTCCTTCTTATTTATC-3' were also designed to insert the *TcVSP* sequence into a C-terminal GFP-fusion plasmid derived from pTREX. All *T. cruzi* sequences were verified by sequencing (Yale DNA Analysis Facility, Yale University, New Haven, Connecticut). *TcVSP-GFP* parasites were used only for localization studies (Fig. 2). In all other studies we used *TcVSP* overexpressing parasites without GFP.

Heterologous expression of TcVSP in *E. coli*

The open reading frame of *TcVSP* (NCBI GenBank nucleotide accession number DQ090844, corresponding to Tc00.1047053503613.60 in TritypDB.org) was amplified by RT-PCR using *Pfu* DNA polymerase and the oligonucleotide primers 5' - GCCATGGCATCTGAAAATAAGGCGACG-3' and 5' - GCTCGAGCACCTCTTCCTTCTTATTATC-3' (NcoI and XhoI restriction sites are underlined). The PCR product of *TcVSP* was cloned into *E. coli* expression vector pET28a. The resulting construct *TcVSP*pET28a, which was confirmed by DNA sequencing, had a C-terminal in-frame fusion of *TcVSP* with a histidine tag (His₆) encoded by the vector. The recombinant construct *TcVSP*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. *TcVSP* gene expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after the culture density reached an OD₆₀₀ of 0.5. The cells were harvested after incubation at 25 °C for 20 h and pellets were washed twice with cold PBS and kept frozen at -80 °C until use.

Bacterial cell pellets were resuspended in 1 × 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 on ice with 30-s intervals (Branson Sonifier 450). After centrifugation at 20,000 *g* for 30 min at 4°C, the supernatant was loaded into 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 TcVSP (rTcVSP) 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 with 50 mM Tris-HCl (pH 7.5) using HiTrap desalting column (Amersham, Piscataway, NJ). The purified rTcVSP was aliquoted and stored at -80°C. The purity of rTcVSP was determined by SDS-PAGE. Protein concentration was determined by the protein assay reagent (Bio-Rad) using bovine serum albumin (BSA) as a standard.

Antibody generation and purification

About 5 mg of rTcVSP protein expressed in *E. coli* (as described above) were affinity-purified using His-Bind Quick 900 Cartridges under denaturing conditions in which 6 M urea was included in all the buffers used. The purified rTcVSP protein was sent to Cocalico Biologicals, Inc. (Reamstown, PA) for production of rabbit polyclonal antiserum. The affinity purification of anti-TcVSP antibody was performed using the cyanogen bromide-activated resin from Sigma. Although these antibodies were useful for immunoelectron microscopy they gave an unspecific pattern in IFA probably because of differences in antigen presentation.

SDS-PAGE and western blot analysis

Electrophoresis was performed as described by Laemmli (Laemmli, 1970) under reducing conditions. Electrophoresed proteins were transferred to nitrocellulose membranes using a Bio-Rad transblot apparatus. Following transfer, the membrane blots were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T) at 4°C overnight. Blots were probed with affinity-purified anti-TcVSP polyclonal antibody (1:5,000 dilution), anti-GFP monoclonal antibody (1:10,000 dilution), anti-TcVP1 monoclonal antibody (1:2,500 dilution), or anti-β-tubulin monoclonal antibody (1:40,000) for 1 h. After washing 5 times with PBS-T, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:20,000), or goat anti-mouse antibody (1:20,000). 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.

Post-translational modifications of TcVSP

For the analysis of the post-translational modifications of TcVSP, wild type epimastigotes or epimastigotes overexpressing *TcVSP* were collected during exponential growth and washed twice with PBS. Parasites were then resuspended in lysis buffer (125 mM sucrose, 50 mM KCl, 4 mM MgCl₂, 0.5 mM EDTA, 20 mM Hepes, pH 7.2, freshly added 1 mM DTT, mammalian protease inhibitor cocktail at a concentration of 1:250 and 0.5% NP-40), and incubated at 4°C for 30 min. An aliquot was taken after lysis for protein quantification. Epimastigote lysates were then incubated with PNGase F or Lambda protein phosphatase. Reaction conditions were as suggested by manufacturers. Samples were incubated in presence or absence of PNGase F or protein phosphatase. After incubations, the epimastigote lysates were immunoblotted using anti-TcVSP antibody. Anti-tubulin was used as loading control.

Mass spectrometry identification

To identify the bands observed in western blots from epimastigotes overexpressing *TcVSP*, total cell lysates of these cells (obtained as described above) were immunoprecipitated using the Seize × Protein G Immunoprecipitation Kit (Pierce Cat.#45210). Briefly, 62 µg of purified anti-TcVSP antibody was applied to Immobilized Protein G and allowed to bind for 1 h at room temperature. Following this, 1.5 mg of total epimastigote lysate was exposed to the gel-bound TcVSP antibody overnight at 4°C. The immunoprecipitated antigen was eluted at acid pH and immediately neutralized. An aliquot of the eluted antigen was tested by western blot analysis and the remaining antigen was separated by SDS-PAGE in a preparative gel. Following Coomassie Blue staining the TcVSP bands around 45 kDa were cut from the gel and sent for mass spectrometry analysis (Complex Carbohydrate Research Center mass spectrometry facility, University of Georgia, Athens, GA).

Pyrophosphatase and polyphosphatase activities

rTcVSP activity was assayed measuring release of P_i using the method of Lanzetta et al (Lanzetta *et al.*, 1979). The standard assay mixtures varied with the substrate used. For PP_i, the standard assay mixtures included 50 mM Tris-HCl (pH 8.5), 3 mM MgCl₂, 100 µM PP_i and 25 ng of purified and desalted rTcVSP protein. For polyP₃, the reaction mixture contained 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.5), 3 mM CoCl₂, 150 µM polyP₃ and 25 ng of rTcVSP. For polyP₇₅ and GP₄, the reaction mixture contained 50 mM MES (pH 6.5), 3 mM ZnCl₂, 100 µM polyP₇₅ or GP₄, and 100 ng of rTcVSP. The reactions were performed in 96-well plates and started by the addition of rTcVSP. After incubation for 10 min at 30°C, the reactions were stopped by the addition of the equal volume of the freshly prepared mixture of 3 parts of 0.045% malachite green and 1 part of 4.2% ammonium molybdate, which was filtered prior to use as described before (Shatton et al., 1983). The absorbance at 660 nm was read using a SpectraMax M2^e plate reader (Molecular Devices, Sunnyvale, CA). The amount of P_i released was determined by comparison with a standard curve. The specific activity of rTcVSP was defined as µmol P_i released/min per mg of protein (µmol/min × mg).

To measure PPase activity in total cell lysates of epimastigotes, the cell lysates were prepared as described under “Cell Culture”. The protein concentration was measured using a Bio-Rad protein assay reagent. The PPase activity was subsequently determined in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 40 µM aminomethylendiphosphonate (AMDP), 100 µM PP_i, and 0.5 µg of cell total lysate. Forty µM AMDP were added to inhibit the vacuolar proton pyrophosphatase (V-H⁺-PPase, TcVP1).

Subcellular fractionation

For the isolation of soluble and membrane-bound fractions for localization studies (Fig. 3B-C), wild type and epimastigotes transfected with GFP/pTREX expression plasmid were collected and washed twice with buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM D-glucose and 50 mM Hepes, pH 7.2) containing a 1:500 dilution of mammalian cell protease inhibitor cocktail (Cat. No. P8340, Sigma, St. Louis). The cell pellets were resuspended in buffer A (homogenates) and sonicated twice for 5 sec at 15% amplitude on ice (Branson Sonifier 450). After centrifugation at $20,000 \times g$ for 10 min, the cell pellets and supernatants were used immediately for western blot analysis and to measure pyrophosphatase activity.

For the determination of total, cytosolic and organellar PP_i (Figs. 5C-5E) wild type or *TcVSP-OE* epimastigotes were collected and washed twice with buffer A with glucose (BAG) by centrifuging at $1,600 \times g$ for 10 min. Parasites were counted and distributed in microcentrifuge tubes (2×10^8 cells per tube). Parasites were then washed once in a hyposmotic lysis buffer (20 mM Hepes, pH 7.3, 100 mM sucrose, and 10 mM sodium fluoride) by centrifugation at $5,000 \times g$ 5 min. After centrifugation, one sample was extracted using 0.5 N perchloric Acid (PCA), incubated in ice for 30 min, and separated for further analysis (Total PP_i). The other samples were subjected to three cycles of freezing and thawing using a solid CO₂/ethanol bath (5 min) and a 37°C water bath (1 min), resuspended in hyposmotic lysis buffer and centrifuged at $20,000 \times g$ for 20 min at 4°C. The supernatant was taken as the cytosolic fraction and the pellet as organellar fraction containing PP_i stored predominantly in acidocalcisomes. Both samples were extracted using 0.5 N PCA and incubated in ice for 30 min. The PCA extracted samples were centrifuged at $12,000 \times g$, at 4°C for 5 min. All extracts were neutralized using 0.6 M KHCO₃, 0.72 M KOH. All fractions were analyzed as described below for PP_i. The efficiency of this fractionation was assessed by testing for pyruvate kinase activity, a cytosolic enzyme in *T. cruzi* (Cazzulo *et al.*, 1989). Pyruvate kinase was detected in a kinetic assay following NADH oxidation by monitoring fluorescence (excitation, 340 nm; emission, 460 nm) using a SpectraMax M2^e plate reader.

Extraction and determination of PP_i, ATP, short chain and long chain polyP levels in epimastigotes

Cells in log phase were harvested and washed twice with buffer A. The PP_i, ATP, and short chain polyP were extracted using 0.5 M perchloric acid (HClO₄) (Ruiz *et al.*, 2001), and the long chain polyP was extracted using glass milk (Molecular Probes) as described by Ault-Riché *et al.* (Ault-Riche *et al.*, 1998).

PP_i was determined by measuring the amount of P_i released upon treatment with *S. cerevisiae* (Sigma) or *Thermococcus litoralis* (New England Biolabs) inorganic pyrophosphatase. Extracts were incubated with 5 units/ml of yeast pyrophosphatase in 50 mM Tris-HCl, pH 7.5, and 6 mM MgCl₂, at 30°C for 30 min, and the released P_i was measured using malachite green as described above. ATP was determined using the ATP determination kit from Molecular Probes according to the manufacture's instructions. PolyP was determined by the amount of P_i released upon treatment with an excess of *S. cerevisiae* exopolyphosphatase 1 (ScPPX1), freshly purified in our laboratory (Ruiz *et al.*, 2001). The intracellular concentration of ATP, was calculated based on the measured intracellular volume of 34.8 µl for 10^9 cells (Rohloff *et al.*, 2003). The concentration of PP_i and polyP in *T. cruzi* epimastigotes was expressed per number of cells used to perform the extractions.

Electron microscopy

About 1×10^8 epimastigotes were harvested and washed twice with PBS. The parasites were fixed with freshly prepared 0.1% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium

cacodylate buffer (pH 7.3) on ice for 1 h. Cells were centrifuged at 14,000 rpm in an Eppendorf centrifuge, infiltrated with 2.3 M sucrose, transferred to specimen freezing supports, and quick frozen by immersion into liquid nitrogen. Supports were then transferred to a cryoultramicrotome (Reichert) and cryosections obtained at a temperature range of -70 to -75 °C. Sections were collected in 2.3 M sucrose with a platinum loop, placed on Formvar/carbon-coated nickel grids, and transferred to 3% BSA in PBS. Samples were blocked with 50 mM ammonium chloride and 3% BSA in PBS. Immunogold labeling was carried out with a purified anti-TcVSP rabbit polyclonal antibody (1:200) and mouse polyclonal antibody against TbVP1 (1:75). After washing, the grids were incubated with 5 nm gold-conjugated goat anti-rabbit IgG secondary antibody (1:100) and 15 nm colloidal gold conjugate goat anti-mouse IgG antibody. Images were acquired on a Megaview III digital camera attached to a Zeiss 900 transmission electron microscope, operating at 80 kV.

For imaging whole epimastigotes, cells were washed with buffer A twice, directly applied to Formvar-coated copper grids, and observed in an energy-filtering Zeiss EM 902 electron microscope operating at 80 Kv. 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., 2007a). Statistical significance was determined by Student's t test. $P < 0.05$ was taken as significant.

Osmotic stress

Briefly, trypomastigotes were washed twice in PBS and resuspended at a density of 4×10^8 cells/ml in isosmotic buffer (64 mM NaCl, 4 mM KCl, 1.8 mM CaCl_2 , 0.53 mM MgCl_2 , 5.5 mM glucose, 150 mM D-mannitol, 5 mM Hepes-Na, pH 7.4, 282 mosmol l^{-1}). Aliquots of 4×10^8 parasites were distributed in 96 well plates and an appropriate volume of hyperosmotic buffer (64 mM NaCl, 4 mM KCl, 1.8 mM CaCl_2 , 0.53 mM MgCl_2 , 5.5 mM glucose, 500 mM D-mannitol, 5 mM Hepes-Na, pH 7.4, 650 mosmol l^{-1}) was added to reach a final osmolarity of 650 mosmol l^{-1} . Osmolarity was determined using an Advanced Instruments 3D3 osmometer. The 96-well plates were read at 550 nm for 10 min using a SpectraMax M2^e plate reader to monitor the changes in cell volume by light scattering (Rohloff et al., 2003). The results were normalized respect to the value of a 3 min pre-reading under isosmotic conditions.

Differentiation of epimastigotes to trypomastigotes

Epimastigotes of stably growing *TcVSP*/pTREX and *GFP*/pTREX transformants were differentiated into intermediate forms or metacyclic trypomastigotes and isolated using a complement selection procedure (Caler et al., 1998). Cultures were allowed to age to the late stationary phase (8-9 days) and observed for the appearance of long slender shaped cells indicative of intermediate forms. Approximately 1×10^7 parasites were removed, washed twice in Dulbecco's phosphate buffer saline (PBS) and used to infect L₆E₉ myoblasts cultures grown in 25 cm² culture flasks with DMEM plus 20% fetal bovine serum. Trypomastigotes were later obtained from infected cell cultures and used for infection assays.

In vivo infection

C57BL/6 (National Cancer Institute, Frederick, MD), C57BL/6-IFN- γ -knockout (The Jackson Laboratory, Farmington, CT) and BALB/c (Harlan Laboratories, Tampa, FL) mice were purchased from the indicated suppliers and maintained at the University of Georgia animal facility in microisolator cages under specific pathogen-free conditions. Tissue culture trypomastigotes (TCT) of the Y, *TcVSP-OE* and CL-TdTomato strains of *T. cruzi* were obtained from passage through Vero cells. Mice were infected i.p. with 5,000 tissue culture trypomastigotes of Y or *TcVSP-OE* strains and sacrificed by CO₂ inhalation at different

time points post infection. For the footpad challenge, mice previously infected with Y or *TcVSP-OE* strains were infected in each pad of both hind feet with 2.5×10^5 trypomastigotes (CL strain) expressing the tdTomato protein. The fluorescence intensity (photons/cm²/sec) was measured as a surrogate of parasite load at the site of infection. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved by the Institutional Animal Care and Use Committee (IUCAC) of the University of Georgia.

In vivo fluorescence imaging

Prior to fluorescence imaging, mice were anaesthetized with 1.5% isoflurane. Mice were placed into the camera chamber, where a controlled flow of 1.5% isoflurane in air was administered through a nose cone via a gas anesthesia system. Footpads of infected mice were imaged at several time points after challenge using the Maestro2 In Vivo Imaging System (CRi, Woburn, MA) with the green filter set (acquisition settings: 560 to 750 in 10 nm steps; exposure time 88.18 ms and 26 2 binning). Collected images were unmixed and analyzed with the Maestro software v2.8.0A.

Assessment of parasite persistence

Mice were immunosuppressed with cyclophosphamide (200 mg/kg/day i.p.) at 2-3 day intervals for a total of 5 or 6 doses. Following immunosuppression, blood was collected via tail vein and the number of parasites was quantified using a Neubauer hemocytometer. Survival was monitored daily. The DNA preparation, generation of PCR standards, and detection of parasite tissue load by real-time PCR was carried out as described previously (Cummings *et al.*, 2003; Bustamante *et al.*, 2008). Skeletal muscle, heart, fat, liver and kidney tissues were collected at various time points and fixed in 10% buffered formalin. Sections (5 µm) from paraffin-embedded tissues were stained with hematoxylin and eosin for histopathological analysis. For the hemoculture experiments, blood from mice immunosuppressed with cyclophosphamide was collected and cultured in LIT medium and the detection of *T. cruzi* was assessed every week for 2 months.

T cell phenotyping

Mouse peripheral blood was obtained by retro-orbital venipuncture, collected in Na citrate solution, and washed in staining buffer (2% bovine serum albumin, 0.02% azide in phosphate buffered saline (PBS)). Whole blood was incubated with the tetramer-PE and the following labeled Abs: anti-CD8 EF450, anti-CD44 FITC, anti-CD127 APC, anti-KLRG1 PECy7 (eBiosciences, San Diego, CA). Cells were also stained with anti-CD4, anti-CD11b, and anti-B220 (Caltag-Invitrogen laboratories, Carlsbad, CA) for use as an exclusion channel. Cells were stained for 45 min at 4°C in the dark, washed twice in PBS, and fixed with 2% formaldehyde. Red blood cells were lysed in a hypotonic ammonium chloride solution after washing twice in PBS. At least 500,000 cells were acquired using a Cyan flow cytometer (DakoCytomation, Fort Collins, CO) and analyzed with FlowJo software (Tree Star, Inc. Ashland, OR). The MHC I tetramer TSKB20 (ANYKFTLV/Kb) was synthesized at the Tetramer Core Facility (Emory University, Atlanta, GA).

Statistical analyses

Statistical comparisons between groups were performed using Student's *t* test (Prism6 GraphPad Software). Differences were considered significant at a level of $p < 0.05$. The number of independent experiments is indicated as n.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Norbert Bakalara for antibodies against TbVP1, Xiang Zhu and Ron Orlando for the mass spectrometry determinations, and Veronica Jimenez for help in the PNGase experiments and in the preparation of extracts for proteomic analysis. This work was supported by grants AI068647 and AI107663 from the U.S. National Institutes of Health.

References

- Ault-Riche D, Fraley CD, Tzeng CM, Kornberg A. Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli*. *J Bacteriol.* 1998; 180:1841–1847. [PubMed: 9537383]
- Baltscheffsky M, Schultz A, Baltscheffsky H. H⁺-PPases: a tightly membrane-bound family. *FEBS Lett.* 1999; 457:527–533. [PubMed: 10523139]
- Bone GJ, Steinert M. Isotopes incorporated in the nucleic acids of *Trypanosoma mega*. *Nature.* 1956; 178:308–309. [PubMed: 13358724]
- Bringaud F, Baltz D, Baltz T. Functional and molecular characterization of a glycosomal PPI-dependent enzyme in trypanosomatids: pyruvate, phosphate dikinase. *Proc Natl Acad Sci U S A.* 1998; 95:7963–7968. [PubMed: 9653123]
- Brown MR, Kornberg A. The long and short of it - polyphosphate, PPK and bacterial survival. *Trends Biochem Sci.* 2008; 33:284–290. [PubMed: 18487048]
- Bustamante JM, Bixby LM, Tarleton RL. Drug-induced cure drives conversion to a stable and protective CD8⁺ T central memory response in chronic Chagas disease. *Nat Med.* 2008; 14:542–550. [PubMed: 18425131]
- Caler EV, Vaena de Avalos S, Haynes PA, Andrews NW, Burleigh BA. Oligopeptidase B-dependent signaling mediates host cell invasion by *Trypanosoma cruzi*. *Embo J.* 1998; 17:4975–4986. [PubMed: 9724634]
- Canavaci AM, Bustamante JM, Padilla AM, Perez Brandan CM, Simpson LJ, Xu D, Boehlke CL, Tarleton RL. In vitro and in vivo high-throughput assays for the testing of anti-*Trypanosoma cruzi* compounds. *PLoS Negl Trop Dis.* 2010; 4:e740. [PubMed: 20644616]
- Cazzulo JJ, Cazzulo Franke MC, Franke de Cazzulo BM. On the regulatory properties of the pyruvate kinase from *Trypanosoma cruzi* epimastigotes. *FEMS Microbiol Lett.* 1989; 50:259–263. [PubMed: 2668108]
- Collins MH, Craft JM, Bustamante JM, Tarleton RL. Oral exposure to *Trypanosoma cruzi* elicits a systemic CD8⁺ T cell response and protection against heterotopic challenge. *Infect Immun.* 2011; 79:3397–3406. [PubMed: 21628516]
- Cummings KL, Tarleton RL. Rapid quantitation of *Trypanosoma cruzi* in host tissue by real-time PCR. *Mol Biochem Parasitol.* 2003; 129:53–59. [PubMed: 12798506]
- Docampo R, de Souza W, Miranda K, Rohloff P, Moreno SN. Acidocalcisomes - conserved from bacteria to man. *Nat Rev Microbiol.* 2005; 3:251–261. [PubMed: 15738951]
- Docampo R, Jimenez V, King-Keller S, Li ZH, Moreno SN. The role of acidocalcisomes in the stress response of *Trypanosoma cruzi*. *Adv Parasitol.* 2011; 75:307–324. [PubMed: 21820562]
- Docampo R, Moreno SN. Acidocalcisomes. *Cell Calcium.* 2011; 50:113–119. [PubMed: 21752464]
- Docampo R, Scott DA, Vercesi AE, Moreno SN. Intracellular Ca²⁺ storage in acidocalcisomes of *Trypanosoma cruzi*. *Biochem J.* 1995; 310:1005–1012. [PubMed: 7575396]
- Espiau B, Lemercier G, Ambit A, Bringaud F, Merlin G, Baltz T, Bakalara N. A soluble pyrophosphatase, a key enzyme for polyphosphate metabolism in *Leishmania*. *J Biol Chem.* 2006; 281:1516–1523. [PubMed: 16291745]
- Fang J, Rohloff P, Miranda K, Docampo R. Ablation of a small transmembrane protein of *Trypanosoma brucei* (TbVTC1) involved in the synthesis of polyphosphate alters acidocalcisome

- biogenesis and function, and leads to a cytokinesis defect. *Biochem J.* 2007a; 407:161–170. [PubMed: 17635107]
- Fang J, Ruiz FA, Docampo R, Luo S, Rodrigues JC, Motta LS, Rohloff P, Docampo R. Overexpression of a Zn^{2+} -sensitive soluble exopolyphosphatase from *Trypanosoma cruzi* depletes polyphosphate and affects osmoregulation. *J Biol Chem.* 2007b; 282:32501–32510. [PubMed: 17827150]
- Go WY, Liu X, Roti MA, Liu F, Ho SN. NFAT5/TonEBP mutant mice define osmotic stress as a critical feature of the lymphoid microenvironment. *Proc Natl Acad Sci U S A.* 2004; 101:10673–10678. [PubMed: 15247420]
- Kollien AH, Grospietsch T, Kleffmann T, Zerbst-Boroffka I, Schaub GA. Ionic composition of the rectal contents and excreta of the reduviid bug *Triatoma infestans*. *J Insect Physiol.* 2001; 47:739–747. [PubMed: 11356421]
- Kornberg, A. On the metabolic significance of phosphorolytic and pyrophosphorolytic reactions. In: Kasha, M.; Pullman, B., editors. *Horizons in Biochemistry*. Academic Press Inc.; New York: 1962. p. 251-264.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
- Lang F. Mechanisms and significance of cell volume regulation. *J Am Coll Nutr.* 2007; 26:613S–623S. [PubMed: 17921474]
- Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. An improved assay for nanomole amounts of inorganic phosphate. *Anal Biochem.* 1979; 100:95–97. [PubMed: 161695]
- Lee HS, Cho Y, Kim YJ, Lho TO, Cha SS, Lee JH, Kang SG. A novel inorganic pyrophosphatase in *Thermococcus onnurineus* NA1. *FEMS Microbiol Lett.* 2009; 300:68–74. [PubMed: 19744243]
- Lemercier G, Espiau B, Ruiz FA, Vieira M, Luo S, Baltz T, Docampo R, Bakalara N. A pyrophosphatase regulating polyphosphate metabolism in acidocalcisomes is essential for *Trypanosoma brucei* virulence in mice. *J Biol Chem.* 2004; 279:3420–3425. [PubMed: 14615483]
- Li ZH, Alvarez VE, De Gaudenzi JG, Sant'anna C, Frasch AC, Cazzulo JJ, Docampo R. Hyperosmotic stress induces aquaporin-dependent cell shrinkage, polyphosphate synthesis, amino acid accumulation, and global gene expression changes in *Trypanosoma cruzi*. *J Biol Chem.* 2011; 286:43959–43971. [PubMed: 22039054]
- Luoto HH, Baykov AA, Lahti R, Malinen AM. Membrane-integral pyrophosphatase subfamily capable of translocating both Na^+ and H^+ *Proc Natl Acad Sci U S A.* 2013; 110:1255–1260. [PubMed: 23297210]
- Maeshima M. Vacuolar H^+ -pyrophosphatase. *Biochim Biophys Acta.* 2000; 1465:37–51. [PubMed: 10748246]
- Malinen AM, Baykov AA, Lahti R. Mutual effects of cationic ligands and substrate on activity of the Na^+ -transporting pyrophosphatase of *Methanosarcina mazei*. *Biochemistry.* 2008; 47:13447–13454. [PubMed: 19053266]
- Malinen AM, Belogurov GA, Baykov AA, Lahti R. Na^+ -pyrophosphatase: a novel primary sodium pump. *Biochemistry.* 2007; 46:8872–8878. [PubMed: 17605473]
- Martin DL, Weatherly DB, Laucella SA, Cabinian MA, Crim MT, Sullivan S, Heiges M, Craven SH, Rosenberg CS, Collins MH, Sette A, Postan M, Tarleton RL. $CD8^+$ T-Cell responses to *Trypanosoma cruzi* are highly focused on strain-variant trans-sialidase epitopes. *PLoS Pathog.* 2006; 2:e77. [PubMed: 16879036]
- Montalvetti A, Rohloff P, Docampo R. A functional aquaporin co-localizes with the vacuolar proton pyrophosphatase to acidocalcisomes and the contractile vacuole complex of *Trypanosoma cruzi*. *J Biol Chem.* 2004; 279:38673–38682. [PubMed: 15252016]
- Moreno SN, Docampo R. Polyphosphate and its diverse functions in host cells and pathogens. *PLoS Pathog.* 2013; 9:e1003230. [PubMed: 23658515]
- Moreno SN, Silva J, Vercesi AE, Docampo R. Cytosolic-free calcium elevation in *Trypanosoma cruzi* is required for cell invasion. *J Exp Med.* 1994; 180:1535–1540. [PubMed: 7931085]
- Oksanen E, Ahonen AK, Tuominen H, Tuominen V, Lahti R, Goldman A, Heikinheimo P. A complete structural description of the catalytic cycle of yeast pyrophosphatase. *Biochemistry.* 2007; 46:1228–1239. [PubMed: 17260952]

- Rao NN, Kornberg A. Inorganic polyphosphate regulates responses of *Escherichia coli* to nutritional stringencies, environmental stresses and survival in stationary phase. *Progr Mol Subcell Biol*. 1999; 23:183–195.
- Rohloff P, Docampo R. Ammonium production during hypo-osmotic stress leads to alkalization of acidocalcisomes and cytosolic acidification in *Trypanosoma cruzi*. *Mol Biochem Parasitol*. 2006; 150:249–255. [PubMed: 17005261]
- Rohloff P, Montalvetti A, Docampo R. Acidocalcisomes and the contractile vacuole complex are involved in osmoregulation in *Trypanosoma cruzi*. *J Biol Chem*. 2004; 279:52270–52281. [PubMed: 15466463]
- Rohloff P, Rodrigues CO, Docampo R. Regulatory volume decrease in *Trypanosoma cruzi* involves amino acid efflux and changes in intracellular calcium. *Mol Biochem Parasitol*. 2003; 126:219–230. [PubMed: 12615321]
- Ruiz FA, Rodrigues CO, Docampo R. Rapid changes in polyphosphate content within acidocalcisomes in response to cell growth, differentiation, and environmental stress in *Trypanosoma cruzi*. *J Biol Chem*. 2001; 276:26114–26121. [PubMed: 11371561]
- Salmon D, Vanwalleghe G, Morias Y, Denoed J, Krumbholz C, Lhomme F, Bachmaier S, Kador M, Gossmann J, Dias FB, De Muylder G, Uzureau P, Magez S, Moser M, De Baetselier P, Van Den Abbeele J, Beschin A, Boshart M, Pays E. Adenylate cyclases of *Trypanosoma brucei* inhibit the innate immune response of the host. *Science*. 2012; 337:463–466. [PubMed: 22700656]
- Sambrook, F.; Fritsch, EF.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*. 2nd. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1989.
- Schatz DM, Murray PK. Cultivation of *Trypanosoma cruzi* in irradiated muscle cells: improved synchronization and enhanced trypomastigote production. *Parasitology*. 1982; 85:115–125. [PubMed: 6750515]
- Scott DA, de Souza W, Benchimol M, Zhong L, Lu HG, Moreno SN, Docampo R. Presence of a plant-like proton-pumping pyrophosphatase in acidocalcisomes of *Trypanosoma cruzi*. *J Biol Chem*. 1998; 273:22151–22158. [PubMed: 9705361]
- Shatton JB, Ward C, Williams A, Weinhouse S. A microcolorimetric assay of inorganic pyrophosphatase. *Anal Biochem*. 1983; 130:114–119. [PubMed: 6135370]
- Sureka K, Dey S, Datta P, Singh AK, Dasgupta A, Rodrigue S, Basu J, Kundu M. Polyphosphate kinase is involved in stress-induced mprAB-sigE-rel signalling in mycobacteria. *Mol Microbiol*. 2007; 65:261–276. [PubMed: 17630969]
- Tammenkoski M, Benini S, Magretova NN, Baykov AA, Lahti R. An unusual, His-dependent family I pyrophosphatase from *Mycobacterium tuberculosis*. *J Biol Chem*. 2005; 280:41819–41826. [PubMed: 16239227]
- Tammenkoski M, Moiseev VM, Lahti M, Ugochukwu E, Brondijk TH, White SA, Lahti R, Baykov AA. Kinetic and mutational analyses of the major cytosolic exopolyphosphatase from *Saccharomyces cerevisiae*. *J Biol Chem*. 2007; 282:9302–9311. [PubMed: 17215253]
- Urbina JA, Moreno B, Vierkotter S, Oldfield E, Payares G, Sanoja C, Bailey BN, Yan W, Scott DA, Moreno SN, Docampo R. *Trypanosoma cruzi* contains major pyrophosphate stores, and its growth in vitro and in vivo is blocked by pyrophosphate analogs. *J Biol Chem*. 1999; 274:33609–33615. [PubMed: 10559249]
- Young TW, Kuhn NJ, Wadeson A, Ward S, Burges D, Cooke GD. *Bacillus subtilis* ORF yybQ encodes a manganese-dependent inorganic pyrophosphatase with distinctive properties: the first of a new class of soluble pyrophosphatase? *Microbiology*. 1998; 144:2563–2571. [PubMed: 9782505]

Fig. 1. rTcVSP activity with different substrates, A-D, and at different pHs, E-H. PP_i and polyP hydrolysis was determined as described under “Experimental procedures” using 100 μM PP_i, A, E, 150 μM polyP₃, B, F, 100 μM polyP₇₅, C, G, or 100 μM GP₄, D, H, at pH 8.5 (PP_i), A, or 6.5 (polyP₃, polyP₇₅, GP₄), B-D, or at different pHs, E-H. Values in the absence of cations were obtained in the presence of 5 mM EDTA. For pH assay, E-H, the experiments were done using Tris-HCl buffer (pH 7.0 - 9.0) and MES buffer (pH 5.0 - 6.5). The cation concentration was varied for PP_i, A, or was 3 mM for the other substrates, B-D, and for the pH optimum determinations, E-H. Values are means ± SEM (n = 3).

Fig. 2.

Fluorescence microscopy and western blot analysis of TcVSP. A, C, and E are DIC images of epimastigotes shown in B, D, and F, respectively. B. TcVSP-GFP fusion protein localizes mainly to the cytosol of live epimastigotes as detected by direct fluorescence. D. TcGFP localizes to the cytosol of live epimastigotes as detected by direct fluorescence. F. Immunofluorescence analysis of epimastigotes incubated with antibodies (1:200) against TcVSP. G, and H. Western blot analyses reveal the expected sizes for GFP-tagged TcVSP (TcVSP-GFP), GFP (TcGFP), endogenous TcVSP and overexpressed TcVSP (*TcVSP-OE*) (arrows) as probed with antibodies against GFP (1:10,000), G, or against TcVSP (1:5,000), H. Molecular mass standards are shown on the right (kDa).

Fig. 3. Immunoelectron microscopy, and western blot analysis and enzymatic activity of subcellular fractions. **A.** TcVSP (arrowheads) localized in the cytosol and acidocalcisomes, where it co-localizes with TcVP1 (arrows). Bars = 100 nm. **B.** epimastigotes were harvested and homogenized as described under “Experimental procedures”. The whole cell homogenates (*H*) were centrifuged at $20,000 \times g$ for 10 min to obtain supernatant (*S*) and pellet (*P*) fractions. The samples were mixed with $2 \times$ Laemmli sample buffer, boiled (TcVSP, GFP) or not boiled (TcVP1, to avoid aggregation of this polytopic membrane protein due to heating) for 5 min, and analyzed by SDS/PAGE. The separated proteins were transferred to nitrocellulose membrane and probed with anti-TcVSP antibody (*left*), anti-GFP monoclonal

antibody (*center*), and anti-TcVP1 monoclonal antibody (*right*). *Lanes MW* indicate the standard protein markers (in *kDa*). C. Specific activity of TcVSP (*left*) and TcVP1 (*right*) measured in the different fractions as described under “Experimental procedures”. *H*, homogenate; *P*, pellet; *S*, soluble fraction. Values are means $\pm$ SEM (n = 3).

Fig. 4. TcVSP overexpression in epimastigotes. **A.** Western blot analysis shows TcVSP is highly expressed in the *TcVSP*/pTREX transformants (*lane 2*). *Lane MW*, protein molecular mass standards (*in kDa*). Total cell lysates (5 μ g protein) of wild type cells (*lane 1*), TcVSP overexpressing cells (*lane 2*), and 42 ng of purified rTcVSP (*lane 3*) from *E. coli* were subjected to 10% SDS-PAGE. The blot was sequentially probed with anti-TcVSP antibody (*upper panel*, 1:5,000) and anti- α -tubulin antibody (1:40,000) as a loading control (*bottom panel*). **B.** Lysates from cells overexpressing TcVSP (*TcVSP*/pTREX) showed a 7-fold higher pyrophosphatase activity than those of wild type cells or cells transfected with the GFP/pTREX plasmid. **C** and **D.** Effect of *TcVSP* overexpression on amastigote growth in

fibroblasts. In vitro infection assays were carried out as described under “Experimental procedures”. Values are means $\pm$ SD (n = 4). Statistical analysis was using Sigma Plot (t test) (* P = 0.0264 panel, C, and (** P = 0.0077 panel, D).

Fig. 5.

Effect of *TcVSP* overexpression on high-energy phosphate compounds. A-C. overexpression of *TcVSP* led to a significant reduction in intracellular short-chain polyP, A, long-chain polyP, B, and total PP_i, C, and no change in ATP, F. D and E. PP_i content of cytosolic and organellar fractions. PP_i was determined in cytosol, D, and 14,000 *g* pellet (acidocalcisome), E, fractions obtained as described under “Experimental procedures” from wild-type and *TcVSP-OE* parasites. Values in A-F are means $\pm$ SD ($n = 3$). Statistical significance was determined by Student’s *t* test. **P* value < 0.01 .

Fig. 6. Morphological changes after overexpression of TcVSP. A. Wild-type epimastigotes showing numerous acidocalcisomes (dark granules). B. Epimastigotes overexpressing TcVSP show larger and apparently less dense acidocalcisomes. Insets show acidocalcisomes at higher magnification. Whole unfixed parasites were observed with an electron microscope equipped with an energy filter. Bars = 5 µm.

Fig. 7.

Overexpression of *TcVSP* led to reduced response to hyperosmotic stress. Relative change in trypanostigotes cell volume under hyperosmotic stress were measured as described under “Experimental procedures” (control, *filled circles*). *TcVSP-OE* cells have significantly reduced shrinkage (*filled triangles*). Values are means $\pm$ SEM ($n = 3$). Statistical significance was determined by Student’s *t* test, **** $P < 0.0001$.

Fig. 8.

Parasitemia and persistence of infection in C57BL/6 mice. A. Evolution of parasitemia after infection of C57BL/6 immunocompetent mice with 5×10^3 wild type and *TcVSP-OE* parasites of the Y strain of *T. cruzi*. The arrows indicate the times of administration of the immunosuppressant cyclophosphamide (days 149, 152, 155, 157 and 160 after infection). Error bars represent means $\pm$ SEM. Quantitative real-time PCR measurement of *T. cruzi* DNA in heart, B, skeletal muscle, C, and kidney, D, of wild type (WT) and *TcVSP-OE* infected mice at 21 days post-immunosuppression. The dashed line denotes the limit of detection.

Fig. 9. Parasitemia and persistence of infection in IFN- γ knockout mice. A. Evolution of parasitemia after infection of C57BL/6 immunodeficient interferon- γ (IFN- γ)-knockout mice with 5×10^3 wild type and *TcVSP-OE* parasites of the Y strain of *T. cruzi*. B. Survival rate of mice described in A. Quantitative real-time PCR measurement of *T. cruzi* DNA in skeletal muscle, C, and fat, D, of *TcVSP-OE* infected mice at 48 days post-infection.

Fig. 10.

Immune response to wild type and TcVSP-OE parasites. A. The MHC-peptide tetramer of the immunodominant TSKB20/Kb epitope was used to detect *T. cruzi*-specific CD8⁺ T cells in the blood of wild type (filled circles) and TcVSP-OE infected mice (filled triangles) during the evolution of the infection. B. Expression of memory maintenance (CD127) and C. antigen stimulation markers in blood on CD44^{hi} CD8⁺ TSKB20-tetramer T cells from wild type and TcVSP-OE infected mice. D. Parasite load at the site of the infection assessed by quantification of the fluorescent signal from mice previously infected with 5×10^3 wild type and TcVSP-OE parasites of the Y strain of *T. cruzi* and challenged in the hind foot pads with 2.5×10^5 *T. cruzi* tdTomato trypanomastigotes.

Table 1

Kinetic parameters of rTcVSP with PP_i, polyP₃, polyP₇₅ and GP₄ as substrates. Experimental conditions were as described under “Experimental procedures”.

Substrates	Cations (3 mM)	pH	$V_{\max}$ ($\mu\text{moles/min} \times \text{mg}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \cdot \text{M}^{-1}$)
PP _i	MgCl ₂	8.5	41.2 $\pm$ 1.7	8.0 $\pm$ 1.7	0.22	2.7 $\times 10^4$
PolyP ₃	CoCl ₂	6.5	12.2 $\pm$ 0.5	16.7 $\pm$ 2.4	0.13	7.8 $\times 10^3$
PolyP ₇₅	ZnCl ₂	6.5	1.5 $\pm$ 0.04	17.4 $\pm$ 1.9	1.65 $\times 10^{-4}$	9.48
GP ₄	ZnCl ₂	6.5	1.9 $\pm$ 0.08	155.4 $\pm$ 14.5	2.2 $\times 10^{-4}$	1.41

Table 2

Characteristics of acidocalcisomes of epimastigotes overexpressing TcVSP

Cell lines	Number of Acidocalcisomes	Mean area (nm ² × 10 ⁴)	Mean diameter (nm)	Absolute volume (× 10 ⁶ nm ³)
Wild-type	48 ± 4	1.9 ± 0.008	155.3 ± 3.2	2.0 ± 0.12
TcVSP-OE	44 ± 4	3.1 ± 0.150	195.1 ± 5.0	4.1 ± 0.30
P value	0.501	< 0.0001	< 0.0001	0.0001

Results are expressed as means ± S.E.M.