Isolation and Characterization of TgVP1, a Type I Vacuolar H\(^{+}\)-translocating Pyrophosphatase from Toxoplasma gondii

THE DYNAMICS OF ITS SUBCELLULAR LOCALIZATION AND THE CELLULAR EFFECTS OF A DIPHOSPHONATE INHIBITOR*†

Received for publication, September 16, 2002, and in revised form, October 23, 2002
Published, JBC Papers in Press, October 30, 2002, DOI 10.1074/jbc.M209436200

Yolanda M. Drozdowicz‡, Michael Shaw, Manami Nishi, Boris Striepen, Helene A. Liwinski, David S. Roos, and Philip A. Rea§

From the Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Here we report the isolation and characterization of a type I vacuolar-type H\(^{+}\)-pyrophosphatase (V-PPase), TgVP1, from an apicomplexan, Toxoplasma gondii, a parasitic protist that is particularly amenable to molecular and genetic manipulation. The 816-amino acid TgVP1 polypeptide is 50% sequence-identical (65% similar) to the prototypical type I V-PPase from Arabidopsis thaliana, AVP1, and contains all the sequence motifs characteristic of this pump category. Unlike AVP1 and other known type I enzymes, however, TgVP1 contains a 74-residue N-terminal extension encompassing a 42-residue N-terminal signal peptide sequence, sufficient for targeting proteins to the secretory pathway of T. gondii. Providing that the coding sequence for the entire N-terminal extension is omitted from the plasmid, transfection of Saccharomyces cerevisiae with plasmid-borne TgVP1 yields a stable and functional translation product that is competent in aminomethylenediphosphonate (AMDP)-inhibitable K\(^{+}\)-activated pyrophosphatase (PP\(_{\text{P}}\)) hydrolysis and PP\(_{\text{P}}\)-energized H\(^{+}\) translocation. Immunofluorescence microscopy of both free and intracellular T. gondii tachyzoites using purified universal V-PPase polyclonal antibodies reveals a punctate apical distribution for the enzyme. Equivalent studies of the tachyzoites during host cell invasion, by contrast, disclose a transverse radial distribution in which the V-PPase is associated with a collar-like structure that migrates along the length of the parasite in synchrony with and in close apposition to the penetration furrow. Although treatment of T. gondii with AMDP concentrations as high as 100 \(\mu\)M had no discernible effect on the efficiency of host cell invasion and integration, concentrations commensurate with the \(I_{50}\) for the inhibition of TgVP1 activity in vitro (0.9 \(\mu\)M) do inhibit cell division and elicit nuclear enlargement concomitant with the inflation and eventual disintegration of acidocalcisome-like vesicular structures. A dynamic association of TgVP1 with the host cell invasion apparatus is invoked, one in which the effects of inhibitory V-PPase substrate analogs are exerted after rather than during host cell invasion.

Vacular-type H\(^{+}\)-translocating inorganic pyrophosphatases (V-PPases)\(^1\) are primary electrogenic proton pumps that derive their energy from the hydrolysis of inorganic pyrophosphate (PP\(_{\text{P}}\)) (1). Long considered to be restricted to plants and certain photosynthetic bacteria, V-PPases have recently been identified in a wide range of organisms, including prokaryotic extremophiles and the kinetoplastid protists Trypanosoma and Leishmania, the causative agents of Chagas’ disease and leishmaniasis, and the apicomplexan protists Plasmodium and Toxoplasma, the causative agents of malaria and toxoplasmosis (reviewed in Ref. 2). The discovery of V-PPases in these parasitic protists has attracted much attention. The seemingly complete absence of V-PPases from their animal hosts has given rise to the exciting possibility that this enzyme might serve as an effective drug target for a number of the diseases caused by these pathogens.

All characterized V-PPases are constituted of a single 75–82-kDa intrinsic membrane protein species that is now known to fall into two structurally and functionally distinct types, I and II. Type I V-PPases, as exemplified by the molecular prototype, AVP1 from Arabidopsis (3), exhibit a near obligate requirement for millimolar K\(^{+}\) for activity. Type II V-PPases, as exemplified by Arabidopsis AVP2 (4), by contrast, share only ~30% sequence identity with their type I counterparts and are insensitive to K\(^{+}\).

A property of all V-PPases characterized to date, regardless of whether they are type I or type II enzymes, which distinguishes them from soluble PPases, is their high sensitivity to competitive inhibition by the 1,1-diphosphonate, aminomethylenediphosphonate (AMDP), and their relative insensitivity to irreversible inhibition by fluoride (5, 6). Originally identified in screens for PP\(_{\text{P}}\), analogs capable of functionally distinguishing V-PPases from soluble PPases and other phosphohydrolases, diphosphonates such as AMDP that contain a heteroatom (NH\(_{2}\) or OH) on the bridge carbon are exquisitely potent V-PPase inhibitors (5). Taking their cue from the finding that protozoal V-PPases are as sensitive to inhibition by AMDP as their plant and photosynthetic bacterial counterparts, several investigators have shown that this compound inhibits the growth of Plasmodium falciparum (7), Toxoplasma gondii (8), and Trypanosoma cruzi (9). Although in all cases the apparent efficacy of

* This work was supported by United States Department of Energy Grant DE-FG02-91ER20055 (to P. A. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Department of Energy/National Science Foundation/United States Department of Agriculture Plant Training Grant Research Fellow during the initial phases of this work.
‡ To whom correspondence should be addressed. Tel.: 215-888-0807; Fax: 215-888-8780; E-mail: parea@sas.upenn.edu.

1 The abbreviations used are: V-PPases, vacuolar-type H\(^{+}\)-pyrophosphatases; AMDP, aminomethylenediphosphonate; HFF, human foreskin fibroblast; RT, reverse transcriptase; RACE, rapid amplification of cDNA ends; ORF, open reading frame; YFP, yellow fluorescent protein; GFP, green fluorescent protein; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy- methyl)propane-1,3-diol.
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AMDP in vitro is markedly lower than its efficacy in vitro, the potential for the development of derivatives of this compound or alternative V-PPase-specific agents for drug purposes is nevertheless evident.

Those plant V-PPases that have been characterized in detail are found primarily in vacuolar and Golgi membranes, where their activity contributes to the transmembrane H⁺ gradient that drives H⁺−/or electrically coupled secondary transport processes (1). By analogy, parallel biochemical and immunological investigations of the V-PPases of trypanosomatid and apicomplexan protists indicate that they are most closely associated with a vacuole-like organelle, the acidocalcisome (8, 10–13). Acidocalcisomes are small, electron-dense vacuolysosome-like acidic compartments, replete with polyphosphates complexed with Ca²⁺, Mg²⁺, and other mineral ions, that are suspected to play a dominant role in Ca²⁺ storage and signaling (reviewed in Ref. 14). The recent demonstration of V-PPases in the membranes binding the contractile vacuoles of Chlamydomonas (15) and Dictyostelium (16) and the remarkable equivalence of the composition of these organelles with acidocalcisomes may also be pertinent to these considerations. Having made this point, it should be stressed that the association of V-PPases with acidocalcisome-like membranes does not preclude their association with other membranes. As indicated by the results of a number of studies of trypanosomatids and apicomplexans, V-PPase-like immunoreactivity is also to be found in the plasma membrane (7, 8, 12, 13, 17) and Golgi system (17), at least under some circumstances.

Several publications have described the preliminary in vitro biochemical characterization of PPase activities associated with membranes prepared from trypanosomatid and apicomplexan protists (reviewed in Ref. 14). However, the molecular basis for these activities has been less well defined. Only a single type I V-PPase gene has been isolated from T. cruzi (18), and in the apicomplexa, although genes for both type I and type II enzymes have been cloned from P. falciparum (19). These restriction fragments were cloned into Bluescript plasmid pKS + and pKS−, respectively, all attempts to elucidate the functional properties of these gene products by heterologous expression have been unsuccessful (7).

In the following we report the isolation and characterization of a type I V-PPase, TgVP1, from the apicomplexan protist, T. gondii, and rigorous analyses of the subcellular localization of the enzyme and of the effects of the V-PPase inhibitor AMDP on parasite morphology both during and after host cell invasion. We demonstrate that, upon heterologous expression in yeast, TgVP1 encodes an intrinsic membrane protein competent in PP−/H⁺ transport that is unique among V-PPases in containing an N-terminal signal sequence sufficient for targeting proteins to the secretory pathway in T. gondii. Furthermore, using affinity-purified V-PPase-specific antibodies, we demonstrate a dynamic pattern of distribution of the V-PPase in invading parasites. Under most conditions, immunofluorescence microscopy of the V-PPase reveals a punctate apical distribution. However, during invasion of the host cell, this immunofluorescence undergoes a dramatic redistribution to assume a collar-like structure at the periphery of the parasite that migrates in synchrony with the penetration furrow as the parasite enters the host cell. Given this association of the V-PPase with the invasion apparatus, it is perhaps surprising that application of even high doses of AMDP to invading parasites has no significant effect on their establishment in the host cell, despite the facility with which lower doses impair intracellular parasite division. These results demonstrate the effects of AMDP at doses much lower than those reported previously and suggest a function for acidocalcisomes in host cell invasion in T. gondii.

MATERIALS AND METHODS

Host Cells and Parasites—T. gondii RH strain tachyzoites were maintained by serial passage in primary human foreskin fibroblast (HFF) cultures grown at 5% CO₂ in bicarbonate-buffered modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated newborn bovine serum (HyClone) and an antibiotic mixture of penicillin, streptomycin, and gentamycin as described (19). The culture medium was replaced with modified Eagle’s medium containing 1% dialyzed fetal bovine serum (Invitrogen) immediately before infection with fibroblasts with parasites.

Cloning Reagents—The PCR primers used for cloning, plasmid construction, RT-PCR, and 5′-RACE are listed in Table I. The T. gondii cDNA pools were prepared from and the 5′-RACE reactions were performed on total RNA and polya(T) mRNA purified from freshly lysed tachyzoites using a SMART RACE Kit (Clontech). DNA sequencing was by dye terminator chemistry using nested oligonucleotide primers.

Isolation of Genomic and cDNA Clones of TgVP1—The genomic and cDNA clones of TgVP1 were isolated by a combination of PCR and standard oligonucleotide hybridization screens of genomic and cDNA libraries. Initially, degenerate primers corresponding to the conserved “universal” V-PPase sequences DNAGGIAE and WDNNAKKYI (primers “UniversalI” and “UniversalII” in Table I) were used in PCRs in which RH genomic DNA or tachyzoite cDNA pools were used as templates. The resulting unique 1028-bp genomic and 593-bp cDNA PCR products were cloned into pGEM-T vector (Promega), sequenced, and used as probes for subsequent parallel screens of an RH genomic library constructed in DASH-II (Stratagene, La Jolla, CA) and a tachyzoite cDNA library (from the AIDS Reference and Reagent Repository, National Institutes of Health, Bethesda, MD).

The longest cDNA clone isolated in the hybridization screens was 1989 bp in length and, by comparison with the published sequences of V-PPases from other sources, appeared to lack the coding sequence for functional enzymes. The largest of three overlapping clones from the hybridization screens of the genomic library yielded two restriction fragments after EcoRI-HindIII digestion, one of 6.2 and another of 3.4 kb, that hybridized at high stringency in Southern analyses with the 1989-bp partial cDNA sequence. These restriction fragments were cloned into the EcoRI-HindIII sites of Bluescript plasmid pKS + (Stratagene) and sequenced. In order to deduce the translation start site of TgVP1 from its genomic sequence, several 5′−RACE and nested RT-PCRs were performed using the tachyzoite cDNA pools as template, and the primer combinations listed in Table I.

Plasmid Construction—The full-length TgVP1 cDNA (GenBank accession number AF320281) was cloned by RT-PCR of T. gondii tachyzoite cDNA pools using Pfu DNA polymerase (Stratagene) and sense and antisense primers (Table I) corresponding to positions 1–20 and 2430–2451, respectively, of the ORF predicted from the TgVP1 genomic sequence (GenBank accession number AF320282). After digestion with Bsal the resulting 2451-bp PCR product was ligated into the multicloning site of pBluescript II-XbaI double-digested yeast expression vector pYES2 (Invitrogen) to generate pYMD23. Yeast expression vectors encoding truncated versions of TgVP1 were generated by PCR amplification of TgVP1 from pYMD23 using Pfu DNA polymerase and sense primers corresponding to the sequence immediately downstream of the predicted signal peptide (residues 121–138 of the TgVP1 cDNA; primer TgVP1.2, Table I) to generate pYMD24, and with sense primers corresponding to the sequence further downstream of this (residues 223–240; primer TgVP1.3, Table I) to generate pYMD25.

The T. gondii fluorescent protein and overexpression plasmids used in this study were constructed in putfB30-YFP/sag-chloramphenicol acetyltransferase vector (20), in which a BgII site separates the 5′−untranslated region of α-tubulin (21) from the P30 signal sequence (22), and an AvrII site separates the P30 sequence from the yellow fluorescent protein-coding sequence (YFP, a derivative of Aequoria victoria green fluorescent protein, GFP). TgVP1 coding sequences were cloned as BamHI-XbaI fragments into BgIII (BamHI-compatible)−AvrII (XbaI-compatible) double-digested vector. For the construction of plasmids pYMD26-27, which contained coding sequences corresponding to the first 84 and 232 amino acid residues of TgVP1 in-frame with the YFP sequence, the TgVP1 coding sequences were amplified by PCR from pYMD23 using the TgVP5′T sense primer in combination with the N-term.1 and N-term.2 antisense primers (Table I). Plasmid pYMD28 containing the full-length TgVP1 cDNA fused to YFP was similarly generated by PCR using primers TgVP5′T and TgVP3′T (Table I). Plasmids pYMD31 and pYMD32, encoding ΔS50N-substituted TgVP1,
were constructed from T. gondii and yeast expression plasmids pYMD25 and pYMD26, respectively, using the QuickChange Site-directed Mutagenesis Kit (Stratagene) and the mutagenic primer "D550N" (Table I).

T. gondii expression plasmids pYMD29-30 and pYMD33-34 were engineered to contain a stop codon upstream of YFP/GFP in the expression vector. Plasmids pYMD29 and -33 were generated by PCR from pYMD25 and pYMD31, respectively, using primers TgVP5'T and TgVP3'Y (Table I). Plasmids pYMD30 and -34 were generated by ligation of the BomHI-Xbal fragments from pYMD25 and pYMD26, respectively, to Bgl II-Bam II double-digested vector.

Plasmid pYES2-AVP1, containing the coding sequence of AVP1, was constructed as described (23).

**Heterologous Expression in Yeast—**Vaccular protease-deficient S. cerevisiae strain BJ5459 (Matu, ara1-52, trpl, lys2-801, leu2-3, hist3-200, pep4:HEIS1, prb1-6R, can1, GAL) transformants containing pYMD25-26, pYMD30, or pYES2-AVP1 were generated by the LiOAc/PEG method, selected for uracil prototrophy, and subjected to denaturation, SDS-PAGE on 10% (w/v) acrylamide gels, electrotransfer, and immunoreaction with antibodies PABHK or PABTK, as described (24). Immunoreactive bands were visualized by ECL (Amersham Biosciences).

**Protein Assays—**Protein was estimated by the method of Bradford (25).

**Light Microscopy—**For light microscopy, HFF cells were grown to confluence on sterilized coverslips in 6-well plates. The confluent cultures were infected with 5 × 10^5 parasites and examined at the times indicated. For the visualization of native fluorescent proteins, the coverslips were mounted in PBS. For the immunofluorescence analyses, parasite-infected cells were fixed in 3% paraformaldehyde and permeated with 0.25% Triton X-100 in PBS. The purified anti-V-PPase sera were used at dilutions of 1:200, and immunoreaction was detected using FITC-conjugated anti-rabbit immunoglobulin (1:1000) (Molecular Probes, Inc.). The anti-MIC3 antibodies (26) in the double-immunolabeling experiments were used at a dilution of 1:1000 and detected using Texas Red-conjugated anti-mouse immunoglobulin (1:1000) (Molecular Probes, Inc.).

Cells expressing YFP fusion proteins were examined using an Axiovert microscope (Carl Zeiss, Inc.), equipped with a single emission filter (505–555 nm; Chroma) and a specific YFP filter (480–495 nm). The images were collected using an interline chip cooled Orca 9545 CCD camera (Hamamatsu).

**Electron Microscopy—**For electron microscopy, infected cells were fixed in situ with a freshly prepared mixture containing 1% glutaraldehyde (made from an 8% stock; Electron Microscopy Sciences, Fort Washington, PA) and 1% osmium tetroxide in 50 mM phosphate buffer (pH 7.4). After adding the fixative at room temperature, the specimens were incubated at 4 °C for 45 min. The samples were rinsed with distilled water to remove excess phosphate before being gently scraped off the Petri dishes with a beveled scraper. Staining was with 0.5% aqueous uranyl acetate for 6–16 h at 4 °C. The samples were dehydrated with acetone and embedded in an Epon-Araldite resin mixture. Ultrathin (50–70-nm thick) sections were cut and stained with uranyl acetate and lead citrate and examined using a Philips 200 electron microscope.

**Assays of Parasite AMDP Sensitivity—**The effects of AMDP on parasite growth were examined in HFF cells grown on coverslips and
infected with tachyzoites at a concentration of 10^7 parasites per ml. The parasites were allowed to invade for 5 min at 37°C before aspirating the medium and replacing it with fresh medium to remove all free parasites. AMDP was added to the medium at the concentrations indicated after allowing 20–30 min at 37°C for the parasites to become established in the host cells. The cultures were incubated in a humidified atmosphere containing 5% CO_2 for 24 h at 37°C, after which time the infected cells were fixed on coverslips in methanol at −20°C. The infected cells remaining in the dishes were processed for electron microscopy as described above.

Parasite replication was assessed by counting the numbers of parasites per parasitophorous vacuole by direct visualization using a Zeiss microscope with phase objectives. To ensure random counting, fields from different sections of the coverslip were counted without prior microscopic examination, and all vacuoles within each field were counted. In all experiments the numbers of parasites per vacuole were determined for between 500 and 700 vacuoles from at least five separate experiments. To examine the effects of AMDP on host cell invasion, freshly emerged tachyzoites were incubated with AMDP for 5–10 min prior to infection. Infected cells were incubated and counted as described above.

Computer Programs—For measurements of the susceptibility of the V-PPase to inhibition by Ca^{2+}, the concentration of free Ca^{2+} ([Ca^{2+}]_\text{free}) was estimated by substitution of the appropriate stability constants into the SOLCON program (a kind gift from Dr. Yale Goldman, Department of Physiology, University of Pennsylvania). The stability constants were obtained from Martell and Smith (27) and Goldman, Department of Physiology, University of Pennsylvania). The stability constants into the SOLCON program (a kind gift from Dr. Yale Goldman, Department of Physiology, University of Pennsylvania). The stability constants were obtained from Martell and Smith (27) and Goldman, Department of Physiology, University of Pennsylvania).

RESULTS

Isolation and Characterization of TgVP1—To screen for genes encoding V-PPases in *T. gondii*, degenerate primers corresponding to the sequences, DNAAGIAE and WDANKKVI (positions 511–518 and 694–701 in AVP1) (32), conserved among all known V-PPases (2), were used as primers for PCR amplification of strain RH genomic DNA and tachyzoite cDNA pools. In so doing a unique 1028-bp genomic product and unique 533-bp cDNA product were isolated, each of which was cloned, sequenced, and determined to be capable of encoding a V-PPase. Both isolates were used as probes for hybridization analyses of this N-terminal extension using SignalP (37) and PSORTII (38) but which is substituted by K in type II V-PPases (2, 4). Of strategic significance is the conservation of motifs and residues known to be characteristic of type I V-PPases from higher plants, and PfVP1 indicate irreversibly abolishes catalytic activity (35, 36); (iii) residues Glu345 and Asp550, corresponding to Glu305 and Asp504 in AVP1, whose substrate-protectable alkylation by maleimides.
TgVP1-mediated PPi hydrolysis is measurable in media containing vacuolar membrane-enriched fraction from BJ5459/pYES2-AVP1 ("AVP1")-transformed S. cerevisiae BJ5459 cells. Plasmid pYMD23 contains the entire coding sequence of TgVP1, inclusive of the N-terminal signal peptide; pYMD24 contains the coding sequence of TgVP1 minus the N-terminal signal peptide (residues 1–41); and pYMD25 contains the coding sequence of TgVP1 minus both the N-terminal signal peptide and the remaining 33 residues of the N-terminal extension (residues 1–75). All lanes were loaded with 5 µg of membrane protein, and the immunoreactive bands were visualized by ECL ("Materials and Methods"). The immunoreactive bands shown were the only ones detected.

(pYMD25). For comparative purposes, yeast were also transformed with pYES2-AVP1 vector.

Expression of TgVP1, measured as the presence of a polypeptide of the appropriate size and immunoreactivity in the vacuolar membrane-enriched fraction from the yeast transformants, is contingent on deletion of the coding sequence for the entire 74-residue N-terminal extension. Vacuolar membrane-enriched vesicles purified from pYMD25-transformed (BJ5459/pYMD25) cells contain an intense PABTK- and PABHK-reactive 75,000 band that reacts with antibody with similar intensity to the corresponding band in the equivalent membrane fraction from pYES-AVP1-transformed BJ5459 cells but is absent from the corresponding fractions from pYMD23- and pYMD24-transformed BJ5459 cells (Fig. 2).

TgVP1-mediated PPi Hydrolysis and PPi-dependent H+ Translocation—The PABTK-reactive polypeptide detected in the vacuolar membrane-enriched fraction from BJ5459/pYMD25 cells catalyzes both PPi hydrolysis and PPi-dependent H+-translocation. When assayed in reaction buffer containing 250 μM NaF to abolish any contribution from contaminating yeast soluble PPase (23), the kinetics of TgVP1-catalyzed PPi hydrolysis are indistinguishable from those of AVP1. The K_m values, PPi concentrations required for maximal activity, and maximal activities of TgVP1 and AVP1 are 34 and 38 μM total PPi, 0.3 mM total PPi, and 0.5–0.6 and 0.6–0.7 μmol/min, respectively, when PPi hydrolysis is assayed in reaction media containing 1.3 mM MgSO_4 (data not shown). As would be predicted for a type I V-PPase, the hydrolytic activity of heterologously expressed TgVP1, like AVP1, is K^+-activated (Fig. 3).

TgVP1-mediated PPi hydrolysis is measurable in media containing choline chloride, albeit at a low level, but replacement of this salt with KCl or potassium gluconate increases activity by 2–3-fold. This behavior is similar to that seen with heterologously expressed AVP1 except that in the latter case K+ increases activity by about 8-fold versus choline (Fig. 3). A similar pattern is seen with PPi-dependent H+-translocation except that the nature of the counter-anion is also important. Substitution of the permeant anion Cl− with the less permeant anion gluconate decreases the extent of both TgVP1- and AVP1-mediated intravesicular acidification by at least 60%, whereas substitution of K+ with choline decreases the extent of intravesicular acidification by at least 90% (Fig. 3). Previous investigations of both endogenous and heterologously expressed type I V-PPases have established that the permeant anion Cl− maximizes PPi-dependent intravesicular acidification by diminishing the magnitude and therefore the stalling action of the inside-positive membrane potential that would otherwise be generated by electrogenic H+ translocation (41).

Sensitivity of TgVP1 to AMDP and Ca2+—TgVP1 and AVP1 are similarly sensitive to inhibition by AMDP (5) but differentially sensitive to inhibition by Ca2+ (Fig. 4). Although the concentration dependences for inhibition of TgVP1- and AVP1-mediated PPi hydrolysis by AMDP superimpose to yield I_50 values of 0.9 and 3.0 μM, respectively (Fig. 4A), TgVP1 is more than 8-fold more sensitive to inhibition by free Ca2+ than AVP1 (Fig. 4B). A total concentration of 1.4 μM, equivalent to a free Ca2+ concentration of 0.15 μM, is sufficient to inhibit TgVP1-mediated PPi hydrolysis by 50%, whereas concentrations of greater than 70 and 1.2 μM, respectively, are required to inhibit AVP1 to the same extent (Fig. 4B).

TgVP1 N-terminal Signal Sequence—To examine whether the putative signal sequence encompassed by the N-terminal extension of TgVP1 is capable of targeting peptides to the secretory pathway in T. gondii, YFP fusion plasmids containing the coding sequences for either the first N-terminal 84 amino acid residues (plasmid pYMD26), which encompass the entire N-terminal signal sequence and cleavage site, or the first 232 residues (pYMD27), which encompass the N terminus, cleavage site, and first predicted transmembrane span of the mature protein, or the entire coding region of TgVP1 inclusive

![Fig. 2](Image 99x560 to 266x737)

**Fig. 2.** Western analysis of antibody PAB_Tk-reactive polypeptides in vacuolar membrane-enriched vesicles purified from pYMD23-, pYMD24-, pYMD25-, or pYES2-AVP1 ("AVP1")-transformed S. cerevisiae BJ5459 cells. Plasmid pYMD23 contains the entire coding sequence of TgVP1, inclusive of the N-terminal signal peptide; pYMD24 contains the coding sequence of TgVP1 minus the N-terminal signal peptide (residues 1–41); and pYMD25 contains the coding sequence of TgVP1 minus both the N-terminal signal peptide and the remaining 33 residues of the N-terminal extension (residues 1–75). All lanes were loaded with 5 µg of membrane protein, and the immunoreactive bands were visualized by ECL ("Materials and Methods"). The immunoreactive bands shown were the only ones detected.

![Fig. 3](Image 318x515 to 562x737)

**Fig. 3.** PPi-dependent H+ translocation and PPi hydrolysis by vacuolar membrane-enriched fractions purified from pYES2-AVP1- and pYMD25-transformed S. cerevisiae BJ5459 (AVP1 and TgVP1, respectively) cells. Intravesicular acidification was monitored with acridine orange in media containing membrane vesicles (80 μg of membrane protein), MgSO_4 (1.3 mM), and 100 mM KCl, potassium gluconate (KGlu), or choline chloride (ChCl). At the times indicated, H+ translocation was initiated by the addition of imidazole-PPi (0.3 mM), and the decrease in fluorescence was measured against time. ΔF% = percentage change in fluorescence (F). PPi hydrolysis by vacuolar membrane-enriched vesicles (3–5 µg of membrane protein) was measured in reaction media (300 μl) containing 100 mM concentrations of the monovalent cations indicated plus 1.3 mM MgSO_4 ("Materials and Methods"). Values shown are means ± S.E. (n = 4).
of the N-terminal extension (pYMD28) were constructed and transfected into tachyzoites (Fig. 5).

In all tachyzoites transfected with pYMD26, YFP fluorescence is observed within dense granules and within the lumen of the parasitophorous vacuole (Fig. 5). In tachyzoites transfected with pYMD27, ~75% of the transfected exhibit sequestration of the YFP fluorescence in the endoplasmic reticulum, whereas the remaining 25% exhibit a punctate fluorescence distribution indicative of the incorporation of YFP into inclusion bodies (Fig. 5). In tachyzoites transfected with pYMD28, the cells divide only rarely and undergo extensive inflation of the vacuole (Fig. 5).

To determine whether the vacuolate, non-dividing phenotype of pYMD28 transfectants might be attributable to the YFP fusion, two constructs, pYMD29 and pYMD30, that either contained or lacked the N-terminal extension but contained a stop codon between the TgVP1 and YFP coding sequences were engineered (Fig. 6). To determine whether the pYMD28 transfectant phenotype might be because of PPa activity associated with the expression product, two of the overexpression constructs, pYMD33 and pYMD34, were engineered to contain a D550N substitution (Fig. 6). It has been established that the same substitution at the equivalent position, residue 504, in AVP1 abolishes catalytic activity whether it is measured as PPi hydrolysis or PPi-dependent H+ translocation (24). Analogous assays of heterologously expressed TgVP1 D550N (construct pYMD32) yield the same result (data not shown). Expression of the TgVP1 sequences in the tachyzoite transfectants was monitored in these experiments by indirect immunofluorescence microscopy using purified peptide-specific antibody PABTK (Fig. 6).

Whereas removal of the YFP tag yields transfectants capable of division at higher frequency, the extents and rates of division of these cells are nevertheless markedly decreased. Moreover, whereas expression of both of the full-length constructs (pYMD29 and -33) results in intense immunostaining that appears to be membrane-associated (Fig. 6), expression of the constructs lacking the N-terminal extension (pYMD30 and -34) results in diffuse immunostaining throughout the cell (Fig. 6). Similar experiments were performed using T. gondii expression plasmid pHFR-P30-GFP/sag-chloramphenicol acetyltransferase vector (42, 48), which contains the dihydrofolate reductase promoter sequence instead of tubulin, but these yielded no observable expression. In no case, did expression of non-functional, D550N-substituted TgVP1 (constructs pYMD33 and pYMD34) abrogate the highly vacuolated, low division frequency phenotype of the transfectants (Fig. 6). Evidently the N-terminal extension of TgVP1 contains a signal sequence sufficient for directing transport to the secretory pathway of T. gondii, but overexpression of the coding sequence of TgVP1, regardless of whether or not the translation product is catalytically active, interferes with cell division and elicits increased vacuolation.

**Subcellular Localization of Endogenous V-PPase**—By having determined the disruptive effects of protein fusion and overexpression techniques in this context, the subcellular localization of TgVP1-related translation products was assessed by using the same purified peptide-specific antibody, PABTK, as used in the experiments presented in Fig. 6. However, as is apparent from the immunofluorescence micrographs shown in Fig. 7, purification of this antibody before use is crucial if the results are to be intelligible. Immunofluorescence microscopy of both free and intracellular tachyzoites using crude preimmune and immune sera and FITC-conjugated secondary antibodies yields remarkably similar results, high intensity fluorescence throughout the tachyzoites (Fig. 7). In striking contrast, when both antisera are affinity-purified against heterologously expressed AVP1 and the specificities of the purification products verified by Western analyses of membranes isolated from S. cerevisiae transformed with either pYES2-AVP1 or empty pYES2 vector, the results are very different. Whereas microscopy using immunopurified preimmune serum discloses little or no immunofluorescence with either intracellular or free tachyzoites, incubation of the same preparations with immunopurified PABTK clearly demonstrates a punctate anterior apical distribution of the antigen (Fig. 7).

**Phase-dependent Changes in Subcellular Distribution**—A striking property of the membrane system with which the V-PPase is associated in the intact parasite is the degree to which it is subject to dynamic phase-dependent changes in organization. Deployment of the same immunopurified antibodies as those used for free and intracellular tachyzoites for studies of trophozoites during host cell invasion reveals that
most of the V-PPase-specific staining assumes a transverse radial distribution soon after the parasite has made contact with the host cell. A collar-like structure is generated that migrates along the length of the parasite in synchrony with and immediately anterior to the apicobasally propagating penetration furrow (Fig. 8). Upon completion of infection, the V-PPase-associated fluorescence disperses before reappearing again at the anterior apex of the intracellular tachyzoite (Figs. 8 and 9).

Although this pattern is reminiscent of the dynamics of microneme protein redistribution during invasion (43), colocalization experiments on these and non-invading parasites using the microneme-specific antibody raised against the secreted microneme protein MIC3 (26) in parallel with reaction with the V-PPase-specific antibody, demonstrate segregation, sometimes diametric segregation, of the two antigens from each other (Fig. 9). MIC3 also propagates as a collar-like structure along the length of the trophozoite during infection, but its distribution is not coincident with that of the V-PPase immediately before and after host cell invasion (Fig. 9).

Effects of AMDP on Cell Invasion and Replication—Knowing that TgVP1 and AVP1, when heterologously expressed in yeast, are similarly sensitive to inhibition by AMDP (Fig. 4) and that reaction of V-PPase-specific antibody with trophozoites during host cell invasion is with a collar-like structure that appears to be associated with the penetration furrow (Fig. 8), we were interested to determine the efficacy of this 1,1-diphosphonate as an inhibitor of cell invasion and/or intracellular parasite replication.

The results of these screens were surprising in that AMDP impairs intracellular parasite replication but exerts little or no effect on host cell invasion. On the one hand, treatment of extracellular trophozoites with AMDP at concentrations as high as 100 μM exerts no discernible effect on either the efficiency of host cell invasion or integration (data not shown). On the other hand, AMDP concentrations as low as 5 μM are sufficient to interfere with intracellular parasite division. Treatment of infected cells with AMDP at concentrations of 5 μM or greater decreases the number of parasites per vacuole after 24 h concomitant with the appearance of irregular parasite masses (Fig. 10). Examination of parasites treated with 10 μM AMDP for 24 h indicates that in many cases daughter cell budding is stalled, leading to the appearance of large irregularly shaped parasites (Fig. 11A). No obvious swelling of the endoplasmic reticulum, nuclear envelope, or Golgi complex occurs, and no large vacuolar spaces are seen in the parasites. Indeed, these parasites contain a full array of normal secretory organelles indicating little or no perturbation of the secretory pathway. Furthermore, the parasite mitochondrion and apicoplast look similar to control parasites. However, in a small number of the parasites (~5%), certain vesicular structures reminiscent of the acidocalcisomes described in trypanosomes (structures typified by the loss of most of their luminal electron density upon double-fixation) lose most of their electron density and undergo swelling and disruption after exposure of the cells to AMDP (Fig. 11B).

**DISCUSSION**

The findings reported here constitute the first molecular and functional characterization of TgVP1, a type I V-PPase from the apicomplexan protist *T. gondii*. It is established that TgVP1 encodes a functional K+-activated, PPi-dependent H+ pump that bears a close resemblance to the canonical type I V-PPases from plants. In addition, it is shown that the V-PPase
has a punctate apical distribution in both free and intracellular tachyzoites at steady state but a dynamic distribution during host cell invasion. Upon initiation of infection of the host cell by *T. gondii*, a collar-like structure with which most of the immuno-detectable V-PPase is associated is formed. This structure, or the V-PPase that is associated with it, moves from the anterior to the posterior of the parasite coincident with propagation of the penetration furrow.

Of the V-PPases that have been defined molecularly to date, TgVP1 is unusual in its possession of an N-terminal signal peptide. With the exception of the pump from *T. cruzi* which like TgVP1 does have one (18), a survey of all available V-PPase sequences from archaea, eubacteria, and plants reveals none, not even the type I V-PPase from *P. falciparum* (7), that contain a putative N-terminal signal peptide. The precise role of this sequence and why protists like *T. gondii* and *T. cruzi* have it and others not is not known, although in the case of TgVP1 it has been shown by the fusion experiments that the N-terminal extension is sufficient to direct polypeptides to the secretory pathway of *T. gondii*.

More sequences are needed to decide this issue, but these findings are consistent with the notion that different parasitic protists have different intracellular V-PPase distributions and that these are determined by the 5’-coding sequences of their genes, namely whether they do or do not specify an N-terminal extension. In the kinetoplastid protist *T. cruzi*, low to intermediate resolution immunological methods localize the V-PPase to acidocalcisomes (11, 44), whereas the results of more recent immunogold electron microscopic analyses of *T. gondii* have been interpreted in terms of localization of the V-PPase to both acidocalcisomes and the plasma membrane (10). In *Plasmodium*, by contrast, in which neither of the V-PPase genes, *PfVP1* nor *PfVP2* (7), encode a polypeptide containing an N-terminal extension, V-PPase immunostaining is predominantly associated with the plasma membrane, an observation supported by the finding that PfVP1 fusions preferentially target to the plasma membrane in trophozoites (7). On the basis of these findings, the sufficiency of the N-terminal extension of TgVP1 for entry of this polypeptide into the secretory pathway, and the presence of this extension not only in TgVP1 but also in its cognate from *T. cruzi*, there is a possibility that targeting of the V-PPase to the acidocalcisome is contingent on initial entry.
Abnormal determined by light microscopy after a further 24 h. The number of parasites per parasitophorous vacuole was media after allowing the parasites 20–30 min to become established in the host cells. The number of parasites per parasitophorous vacuole was determined to be necessary to maximize monospecificity, the differences between the results presented here and those published elsewhere are explicable in terms of the different cross-reactivities of the antibody preparations employed, namely that unless the antisera used (antisera generated by other investigators are explicable in terms of the different cross-reactivities of the antibody preparations employed, namely that unless the antisera used (antisera generated by other investigators are explicable in terms of the different cross-reactivities of the antibody preparations employed, namely that unless the antisera used (antisera generated by other investigators are explicable in terms of the different cross-reactivities of the antibody preparations employed, namely that unless the antisera used (antisera generated by our laboratory (23) in all cases) are not first carefully immunopurified, they react relatively indiscriminately with parasitic protists. We, like Rodrigues et al. (8), observe plasma membrane immunostaining as well as a punctate and diffuse staining throughout the cell when crude antiserum is employed. However, if the monospecificity of the antiserum is maximized by immunoaffinity purifying it against heterologously expressed AVP1, only the structure-specific focused staining we describe here is observed. The implication is clear, the antisera raised against peptide sequences TKAADVGLALGKIE and HKAAVIGDTIGDPLK from AVP1, which have since proven to contain universal V-PPase antibodies (2), also contain antibodies that react with other parasite antigens. Because these antibodies were originally intended to be used in plants and yeast, they were raised in New Zealand White rabbits whose preimmune and immune sera were prescreened against these organisms, not parasitic protists.

What cannot be determined from the investigations we describe here is whether the immunofluorescence seen is solely attributable to TgVP1 or is inclusive of other V-PPases. The antisera used in these studies were raised against peptides that are conserved among all V-PPases identified to date and, as such, do not distinguish type I from type II V-PPases (2). It was disappointing but none of the strategies applied in an attempt to circumvent this limitation were fruitful. Overexpression of TgVP1 fusions, for instance YFP fusions, consistently yielded aberrant nondividing cells, and all of our attempts at isolating a type II sequence from T. gondii were negative. There is a possibility that T. gondii does not contain a type II equivalent, in which case the immunolocalizations obtained are indeed exclusively attributable to TgVP1, but it is more likely, as is the case for P. falciparum (7), that the levels of the type II V-PPase transcript are low, perhaps undetectable in the tachyzoite and bradyzoite stages that were screened. Because of this it is not known if the dynamic distribution of immunostaining is specific to a particular V-PPase or is a property of acidocalcisomes in general. Confirmation or refutation of these two alternatives will have to await the results of further screens, completion of the Toxoplasma genome sequencing project, and/or expansion of the corresponding EST database.

The functional significance of the collar-like structure to which the V-PPase immunolocalizes during host cell invasion is intriguing. Despite the seeming similarity of the distribution of the V-PPase antigen with that of micronemes, the lack of colocalization of this enzyme with the microneme MIC3 antigen dispels the notion of a micronemal localization. Analogously, equivalent experiments using antibodies raised against rhoptry and dense granule antigens show little or no colocalization with the V-PPase (data not shown). The most logical
earlier studies demonstrated an anteriorly displaced concentra-
tion, similar to that found for the V-PPase in this study (46) and that
biochemical investigations of the acidocalcisome-like intracel-
lular stores (48), it is conceivable that acidocalcisomes serve as a
source of this Ca^{2+}. Acidocalcisomes may thereby provide the
Ca^{2+} required for activating the calmodulin-dependent myosin
light chain kinases that regulate the actomyosin motor that
governs parasite motility and host cell invasion (46). If this
were the case, the V-PPase would provide the motive force for
acidocalcisomal Ca^{2+} accumulation by H^{+}/Ca^{2+} antiport.

The finding that even high levels of AMDP do not block host
cell invasion by T. gondii despite the dynamic pattern of V-
Pase immunofluorescence during this process was unexpected.
In retrospect, however, it might be expected that in biochemical investigations of the acidocalcisome-like intracel-
lular structures in trypanosomatids and P. falciparum, which
react with the same antisera as used in this study, have shown
these structures to release Ca^{2+} upon treatment with V-ATPase inhibitors and/or AMDP (12, 49). Thus, the inactiv-
ity, not the activity, of acidocalcisome-associated H^{+}-pumps
like the V-PPase may promote Ca^{2+} release from these org-
anelles, in which case AMDP would be expected not to inter-
ference with host cell invasion. It may be instructive to consider,
given how sensitive heterologously expressed TgVP1 is to in-
hibition by Ca^{2+}, that Ca^{2+} release itself may abrogate V-PPase activity, so diminishing or eliminating futile PP,
NNH^{+} gradient-dependent organellar Ca^{2+} uptake during
host cell invasion.

As an extension of our studies of the in vitro sensitivity of heterologously expressed TgVP1 to inhibition by AMDP and the
studies made by ourselves and others (7–9) of the in vitro
sensitivity of parasitic protists to this and other 1,1-diphos-
phatases, we have explored the morphological consequences to
T. gondii of exposure to this agent. As a result, we have deter-
mined that the toxic action of AMDP is evident at concentra-
tions lower than reported previously. Rodrigues et al. (8), for
instance, by employing a global drug assay based on measure-
ments of [3H]hypoxanthine incorporation, reported that concen-
trations of AMDP as high as 50 μM did not inhibit tachyzoite
proliferation; indeed, 200 μM was not sufficient for 50% inhibi-
tion. We, in contrast, by counting the number of parasites per
vacuole and examining their ultrastructure, have determined
that AMDP concentrations as low as 5 μM (concentrations
commensurate with the K_{50} for the in vitro inhibition of TgVP1)
interfere with parasite replication. AMDP concentrations of
5–10 μM and above block or stall cell replication but not cell
growth such that the formation of highly enlarged tachyzoites
containing multiple nascent daughter cells is apparent. The
effects of AMDP are accompanied by extensive enlargement of
the parasite nucleus which becomes highly lobed and the
inflation and/or disruption of acidocalcisome-like vesicular
structures.

The apparent absence of V-Pases from vertebrates and their
likely involvement in energy conservation and membrane
transport make these enzymes potential targets for the devel-
opment of antiprotozoal agents. Notwithstanding these hopes
and needs, it should be appreciated that care is required when
interpreting the antiparasitic effects of AMDP. Two consider-
atations are crucial. First, the efficacy AMDP in vivo is usually
less than its efficacy in vitro, which likely reflects its bulky
anionic character and slow permeation of membranes. Second,
the greater intrinsic sensitivity of V-Pases versus soluble
PPases and other phosphohydrolases to AMDP is in part attri-
butable to the K_{50} values of V-Pases, which are at least an
order of magnitude greater those of soluble PPases (5, 41).
Therefore, the specificity of AMDP and other PP analogs as
competitive inhibitors will be critically dependent on the PP,
concentration prevailing in the compartment in which the in-
hibitor exerts its effects.

Acknowledgments—We thank Dr. Lewis Tilney for the use of the EM
capabilities and Dr. Cynthia He for enthusiastic help with many of the
immunofluorescence studies described.

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Isolation and Characterization of TgVP1, a Type I Vacuolar H\textsuperscript{+}-translocating Pyrophosphatase from Toxoplasma gondii: THE DYNAMICS OF ITS SUBCELLULAR LOCALIZATION AND THE CELLULAR EFFECTS OF A DIPHOSPHONATE INHIBITOR
Yolanda M. Drozdowicz, Michael Shaw, Manami Nishi, Boris Striepen, Helene A. Liwinski, David S. Roos and Philip A. Rea

J. Biol. Chem. 2003, 278:1075-1085,
doi: 10.1074/jbc.M209436200 originally published online October 30, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209436200

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