A plasma membrane-type Ca^{2+} -ATPase co-localizes with a vacuolar H^{+} -pyrophosphatase to acidocalcisomes of *Toxoplasma gondii*

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Ca^{2+} -ATPases are likely to play critical roles in the biochemistry of *Toxoplasma gondii*, since these protozoa are obligate intracellular parasites and the Ca^{2+} concentration in their intracellular location is three orders of magnitude lower than in the extracellular medium. Here, we report the cloning and sequencing of a gene encoding a plasma membrane-type Ca^{2+} -ATPase (PMCA) of *T. gondii* (*TgA1*). The predicted protein (*TgA1*) exhibits 32–36% identity to vacuolar Ca^{2+} -ATPases of *Trypanosoma cruzi*, *Saccharomyces cerevisiae*, *Entamoeba histolytica* and *Dictyostelium discoideum*. Sequencing of both cDNA and genomic DNA from *T. gondii* indicated that *TgA1* contains two introns near the C-terminus. A hydropathy profile of the protein suggests 10 transmembrane domains. *TgA1* suppresses the Ca^{2+} hypersensitivity of a mutant of *S. cerevisiae* that has a defect in vacuolar Ca^{2+} accumulation. Indirect immunofluorescence and immunoelectron microscopy analysis indicate that *TgA1* localizes to the plasma membrane and co-localizes with the vacuolar H^{+} -pyrophosphatase to intracellular vacuoles identified morphologically and by X-ray microanalysis as the acidocalcisomes. This vacuolar-type Ca^{2+} -ATPase could play an important role in Ca^{2+} homeostasis in *T. gondii*.

Keywords: acidocalcisome/calcium ATPase/*Toxoplasma* vacuolar pyrophosphatase/volutin

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite of humans and animals that has emerged as a major opportunistic pathogen in people with AIDS. Infection with *T. gondii* is usually asymptomatic and results in the formation of dormant encysted bradyzoites that remain in the brain and other tissues for life. Only the developing fetus and the immunosuppressed patient are at substantial risk of severe disease. The tachyzoite is the rapidly growing asexual form that is also seen in acutely infected animals.

Ca^{2+} signaling has been shown to play a key role in the process of mammalian cell invasion by *T. gondii*. An increase in the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) of tachyzoites occurs upon invasion (Vieira and Moreno,

2000), and pretreatment of tachyzoites with an intracellular Ca^{2+} chelator (BAPTA/AM) to prevent the increase in $[\text{Ca}^{2+}]_i$ results in an inhibition of cellular invasion (Vieira and Moreno, 2000). Recent studies (Carruthers and Sibley, 1999; Carruthers *et al.*, 1999) have also demonstrated that agents able to increase $[\text{Ca}^{2+}]_i$ of tachyzoites were able to stimulate microneme secretion, suggesting a role for Ca^{2+} in this process that is essential for penetration of *T. gondii* into host cells (Carruthers and Sibley, 1997, 1999; Carruthers *et al.*, 1999). Unlike mammalian cells, *T. gondii* possesses a great proportion of its intracellular Ca^{2+} in an acidic compartment named the acidocalcisome (Moreno and Zhong, 1996). Acidocalcisomes were first described in trypanosomatids (Vercesi *et al.*, 1994; Docampo *et al.*, 1995; Lu *et al.*, 1997) and have recently been postulated to be similar to the organelles described historically as volutin granules or polyphosphate bodies in different microorganisms (Docampo and Moreno, 1999). Acidocalcisomes in trypanosomatids are characterized by their high electron density, high content of polyphosphates, calcium, magnesium, sodium and zinc (Scott *et al.*, 1997; Urbina *et al.*, 1999), and a number of pumps and exchangers in their limiting membrane, among them a Ca^{2+} -ATPase, a vacuolar H^{+} -ATPase, a $\text{Na}^{+}/\text{H}^{+}$ exchanger and a vacuolar H^{+} -pyrophosphatase (V- H^{+} -PPase) (Scott *et al.*, 1995; Vercesi and Docampo, 1996; Lu *et al.*, 1998; Scott *et al.*, 1998; Rodrigues *et al.*, 1999a,b). Morphologically similar structures have been described in *T. gondii* and termed black granules, and have been postulated to be the acidocalcisomes (Bouchot *et al.*, 1999). In addition, the presence of a V- H^{+} -ATPase and a V- H^{+} -PPase located in intracellular vacuoles has been described in *T. gondii*, but the vacuoles were not identified at the electron microscope level (Moreno *et al.*, 1998; Rodrigues *et al.*, 2000).

This study reports the identification in *T. gondii* of the *TgA1* gene, which encodes a protein with homology to mammalian plasma membrane Ca^{2+} -ATPases (PMCA) and with characteristics that place it in the novel category of Ca^{2+} -ATPases formed by the acidocalcisomal Ca^{2+} -ATPase of *Trypanosoma cruzi* (Lu *et al.*, 1998) and the vacuolar Ca^{2+} -ATPases described in *Saccharomyces cerevisiae* (Cunningham and Fink, 1994), *Dictyostelium discoideum* (Moniakakis *et al.*, 1995) and *Entamoeba histolytica* (Ghosh *et al.*, 2000). Indirect immunofluorescence and immunoelectron microscopy analysis suggest that the product of this gene (*TgA1*) is associated not only with the plasma membrane but also with the acidocalcisomes, where it co-localizes with the V- H^{+} -PPase. The gene is expressed at high levels in both tachyzoites and bradyzoites and is able to complement functionally the *PMCI* gene, encoding the vacuolar Ca^{2+} -ATPase of *S. cerevisiae*.

Results

Cloning and characterization of a Ca^{2+} -ATPase gene

Since *T.gondii* acidocalcisomes (Moreno and Zhong, 1996) are physiologically similar to the same organelles present in *T.cruzi* (Docampo *et al.*, 1995), we looked for the presence of a gene encoding a Ca^{2+} -ATPase with homology to the Ca^{2+} -ATPase present in acidocalcisomes of *T.cruzi* (Lu *et al.*, 1998). Degenerate oligonucleotides corresponding to two conserved domains in Ca^{2+} -ATPases, a phosphorylation site and a site involved in ATP binding (Allen and Green, 1976; Pick and Bassilian, 1981), were used to amplify, by PCR, specific sequences from *T.gondii* genomic DNA. The PCR products were cloned and sequenced. Analysis of the deduced partial amino acid sequence of these clones revealed that a 1.4 kb PCR clone had the best score of sequence identity (50.2%) and similarity (56.6%) with the acidocalcisomal Ca^{2+} -ATPase described in *T.cruzi* (Lu *et al.*, 1998).

To obtain the complete gene, this PCR clone (*TgA1.4*) was used as a probe to screen *T.gondii* genomic and cDNA libraries. Screening of 3×10^5 plaques from the genomic library yielded 51 positive clones. Mapping and sequencing of five clones revealed an 8.5 kb fragment containing a complete open reading frame (ORF), designated *TgA1*, with 4215 nucleotides. Interestingly, this ORF was interrupted by introns near the C-terminal region. The DNA sequence of the ~1.4 kb PCR product was identical to the corresponding region of the gene obtained from the lambda EMBL3 genomic library. Screening of 5×10^5 plaques from the cDNA library with *TgA1.4* yielded 12 positive cDNA clones, $\lambda\text{c}1$ to $\lambda\text{c}12$. Restriction enzyme digestion analysis indicated similar patterns but clone size varied from ~0.8 to ~5.4 kb. Four cDNA clones ($\lambda\text{c}1$, $\lambda\text{c}4$, $\lambda\text{c}7$ and $\lambda\text{c}10$) were selected for sequence analysis. Sequence data indicated that there were identical overlapping areas in all four clones.

Comparison of the genomic sequence with the cDNA sequence revealed that the ORF of *TgA1* contains three exons interrupted by two introns. The exons ranged in size from 320 to 3853 bp. The introns ranged between 330 and 479 bp with the 5'-G/GTRAGY and 3'-(Y)nAG/G conserved splice-site signals typical of eukaryotic nuclear introns (Breathnach and Chambon, 1981; Nagel and Boothroyd, 1988).

According to the consensus translational initiation sequence 'gNCAAAaATGg' for *T.gondii* genes (Seeber, 1997), which is similar to the Kozak sequence (GCCA/GCCATGG) of higher eukaryotes (Kozak, 1989), and the initiation codon ATG predicted, the ORF codes for a protein of 1405 amino acids with a predicted molecular mass of 152 827 Da (Figure 1). The first nucleotide of the longest clone, $\lambda\text{c}7$ (5424 bp), was located 109 nt upstream of the start codon (base 2998). The exact transcription start site was not mapped. A computer-aided database search of nucleotides 1–2900 did not reveal eukaryotic promoter consensus sequences. However, eight sequences with nucleotides cgtct/gct/a (*cis*-acting elements) were identified (Mercier *et al.*, 1996) within nucleotides 1–2500.

Examination of the sequence of 1097 bases flanking *TgA1* at the 3'-end of the ORF revealed some interesting features. The consensus poly(A) signal found in plants and

higher eukaryotes, AATAAA, was not present. However, a related motif, ATAAA, was found twice, at 92 and 981 bases from the stop codon (bases 8225 and 9114), and a motif AATAAG was found 253 bases downstream of the TGA codon (base 8487). Three (TA)₈ strings located at 486, 707 and 789 bases downstream of the stop codon (bases 8620, 8840 and 8922) were identified.

In order to confirm the transcription of the *TgA1* gene and determine the sequence of the 5'-end of the transcript, RT-PCR was performed as described in Materials and methods. Sequence analysis of the RT-PCR products (bands of 540 and 428 bp) indicated that they were derived from the *TgA1* gene and that the predicted initiation codon of the ORF was preceded by 223 bp of 5'-untranslated sequence.

Southern blotting was performed with *TgA1.4* as a probe to confirm the presence of this gene in the *T.gondii* genome (Figure 2A). Genomic DNA from *T.gondii* was digested with several restriction enzymes (selected to demonstrate genome copy number) and hybridized at high stringency with the *TgA1.4* probe. The different restriction enzymes used produced single bands, which varied in size (Figure 2A, lanes 1–5), suggesting that *TgA1* is present as a single copy gene in the haploid *Toxoplasma* genome. No band was detected when bovine turbinate (BT) cells were used as control (Figure 2A, lane 6).

Structure of the coding region and genomic organization of *TgA1*

Analysis of the *TgA1* amino acid sequence (Figure 1) showed that this gene product contains all the conserved subdomains and invariant residues found in other P-type ATPases, such as the phosphorylation and ATP-binding domains (Allen and Green, 1976; Pick and Bassilian, 1981). Hydropathy analysis of the deduced amino acid sequence (Figure 1) revealed a profile very similar to those of other calcium pumps containing 10 transmembrane domains (dashed lines above *TgA1* sequence in Figure 1). As occurs with the vacuolar Ca^{2+} -ATPases described for *T.cruzi* (Lu *et al.*, 1998), *S.cerevisiae* (Cunningham and Fink, 1994), *D.discoideum* (Moniakis *et al.*, 1995) and *E.histolytica* (Ghosh *et al.*, 2000), a TFASTA search of protein databases showed that *TgA1* was closely related to the PMCA, with 37% identity (56% similarity) to the PMCA from human erythrocytes (Strehler *et al.*, 1990). It also had 32–36% identity and 53–55% similarity over its entire length with the sequences of the vacuolar Ca^{2+} -ATPases of *T.cruzi* (Lu *et al.*, 1998), *S.cerevisiae* (Cunningham and Fink, 1994) and *D.discoideum* (Moniakis *et al.*, 1995), respectively, and had 25–29% identity to sarcoplasmic (endoplasmic) reticulum-type Ca^{2+} -ATPases (SERCA) and 22–26% identity to Na^+ , K^+ -ATPases from different species (Shull *et al.*, 1986; Sverdlov *et al.*, 1987). An alignment of the vacuolar Ca^{2+} -ATPases with the *T.cruzi*, *S.cerevisiae* and *D.discoideum* sequences is shown in Figure 1 using CLUSTAL W (Thompson *et al.*, 1994). *TgA1* contains two potential N-glycosylation sites, Asn-Ser-Thr and Asn-Phe-Thr, indicated with a dash above the alignment in Figure 1. *TgA1* lacks the conserved amino acid sequence associated with calmodulin binding that is found near the C-terminus of all mammalian PMCA isoforms (Strehler, 1991), as is also the case for the vacuolar Ca^{2+} -ATPases

Fig. 1. Alignment of different Ca^{2+} -ATPases. CLUSTAL W alignment (Thompson *et al.*, 1994) of Ca^{2+} -ATPases from *T. gondii* (DDBJ/EMBL/GenBank accession No. AF151371), *T. cruzi* (U70620) and *S. cerevisiae* (U03060). Similar residues are shaded. Amino acid residues not present within other sequences are denoted by dashes. The ATP-binding and autophosphorylation domains employed in the design of degenerate oligonucleotides for PCR are underlined, the predicted transmembrane domains in the *T. gondii* sequence are indicated by dashed lines above the alignment. The potential N-glycosylation sites are indicated with a dash above the alignment.

from other eukaryotic organisms, such as *S. cerevisiae* Pmc1p (Cunningham and Fink, 1994) and *D. discoideum* PAT1 (Moniak *et al.*, 1995). Like PAT1 of *D. discoideum* (Moniak *et al.*, 1995) and *tca1* of *T. cruzi* (Lu *et al.*, 1998), TgA1 also has a long extension of ~100 amino acids after transmembrane domain 10, which is absent in Pmc1p.

Expression of TgA1 in tachyzoite and bradyzoite forms of *T. gondii*

Northern blot analysis showed a single ~5.2 kb transcript in tachyzoite and bradyzoite forms of *T. gondii* (Figure 2B, upper panel). Analysis of the ~5.2 kb band by densitometry indicated that the TgA1 transcript is expressed at similar levels in tachyzoites and bradyzoites. Bands obtained after hybridization with a PCR product for the *TUB1* gene, which is expressed at similar levels in tachyzoite and bradyzoite forms of *T. gondii* (Nagel and Boothroyd, 1988) (Figure 2B, lower panel), were used as reference controls.

To detect the TgA1 gene product, antibodies were raised against a protein of 255 amino acids within the large cytosolic loop, fused to a His₆ tag, and purified as described in Materials and methods. This region was chosen because it is the least conserved region of all known Ca^{2+} -ATPases. Total cell lysates prepared from tachyzoite and bradyzoite forms of *T. gondii* were subjected to western blot analysis with the affinity-purified antibodies. These antibodies detected a single band of ~135 kDa, close to the predicted molecular mass of TgA1 (Figure 3, lanes 4–6). A 33 kDa His₆-TgA1 fusion protein was recognized by anti-TgA1 (Figure 3, lane 7). No

background staining was observed when pre-immune serum was used as a control (Figure 3, lanes 1–3).

Functional complementation of the *PMC1* gene of *S. cerevisiae* with TgA1

Saccharomyces cerevisiae K665 with deletion of the genes encoding the high-affinity Ca^{2+} -ATPase and low-affinity $\text{Ca}^{2+}/\text{H}^{+}$ antiporter (*PMC1* and *VCX1*) is intolerant of high Ca^{2+} in the growth medium (Cunningham and Fink, 1994). Since the *T. gondii* TgA1 gene encodes a vacuolar-type Ca^{2+} -ATPase with homology to *PMC1*, we investigated whether complementation of the *vcx1 pmc1* yeast mutants with the TgA1 gene could suppress their Ca^{2+} hypersensitivity. Figure 4A and B shows that transformation of the *vcx1 pmc1* K665 strain with pYES2-TgA1 restored growth on high Ca^{2+} almost completely, thus suggesting the function of TgA1 as a vacuolar Ca^{2+} -ATPase in these mutants. K665 was transformed with a control vector (K665pYES2) or a vector containing the entire ORF of *T. gondii* TgA1 (K665pYES2-TgA1). Strain K661 has the *PMC1* gene (Cunningham and Fink, 1994) and thus served as positive control (Figure 4A and B).

Localization of *T. gondii* Ca^{2+} -ATPase

We investigated the localization of the Ca^{2+} -ATPase in *T. gondii* by immunocytochemistry with the antibodies described above. The reaction of these antibodies in the tachyzoite and bradyzoite forms of *T. gondii* as revealed with fluorescein-labeled secondary antibodies was of variable intensity. We observed strong labeling in intracellular vacuoles and a weak labeling of the cell surface (Figure 5A and F). Live cells were not labeled with these

Fig. 2. Southern and northern analysis of *TgA1*. (A) Southern blot analysis of the *TgA1* gene in genomic DNA from *T. gondii*. Total genomic DNA (10 µg/lane) was digested with various restriction enzymes and analyzed as described in Materials and methods. Size markers are indicated. *Trypanosoma gondii* DNA digested with the following restriction enzymes: lane 1, *EcoRI*; 2, *BamHI*; 3, *HindIII*; 4, *PstI*; 5, *BglII*. Control BT cell's DNA (10 µg/lane) was digested with *EcoRI* and *BamHI* (lane 6). The blots were hybridized with the ³²P-labeled 1.4 kb PCR product (*TgA1.4*) and washed at high stringency. (B) Expression of *TgA1* mRNA in tachyzoite (lanes 1 and 2) and bradyzoite (lane 3) forms of *T. gondii*. Upper panel, poly(A)⁺ RNA (3.7 µg/lane) was electrophoresed, blotted, and probed at high stringency with a ³²P-labeled probe corresponding to the entire *TgA1* ORF. Size markers are indicated on the right. Approximately equal amounts of RNA were observed in the three lanes under UV light. Lower panel, the membranes were stripped and reprobed with a ³²P-labeled PCR fragment of the *TUB1* gene from *T. gondii* as control. Exposure times were 3 days (upper panel) and 4 h (lower panel) at -80°C. Lane 1, tachyzoites, RH strain; lane 2, tachyzoites, ME49 strain; lane 3, bradyzoites, ME49 strain.

antibodies (data not shown). No fluorescence was observed in control parasites incubated only in the presence of the secondary fluorescein-labeled goat anti-rabbit IgG (data not shown) or in the presence of the pre-immune serum (Figure 5C). A monoclonal antibody against BAG-5, a bradyzoite-specific antigen (Weiss *et al.*, 1995, 1996), was used as bradyzoite marker (Figures 5H and 6C and D). No labeling was seen in tachyzoites when incubated with the monoclonal antibody against BAG-5 (Figure 5D). Similarly, the localization of *TgA1* was observed in both tachyzoite (Figure 6E) and bradyzoite (Figure 6B and D) forms of *T. gondii* by using confocal laser scanning microscopy analysis, consistent with the results of the northern and western blot analysis.

In order to analyze in more detail the structures labeled with the antibodies, immunoelectron microscopy was performed on thin sections of parasites embedded in the hydrophilic resin Unicryl. The results obtained confirmed that gold particles were seen in cytoplasmic vacuoles that appeared empty (Figure 7A–C, arrows, 10 nm gold particles), a characteristic feature of acidocalcisomes (Docampo and Moreno, 1999), and on the cell surface (Figure 7A, arrows), consistent with the results of immunofluorescence. Very few gold particles were observed in cytoplasmic structures.

Co-localization studies with the antibody against the Ca²⁺-ATPase (Figure 7A–C, 10 nm gold particles, arrows), and a polyclonal antibody that recognizes a peptide of 28 amino acids in the C-terminal region of the V-H⁺-PPase of *T. cruzi* [Figure 7A–C, arrowheads, 20 (A) or 15 nm (B and C) gold particles], confirmed the presence of both enzymes in the acidocalcisomes. Figure 7E, upper panel, shows that a single protein band with a molecular mass identical to that of the *T. cruzi* epimastigotes (Ep) V-H⁺-PPase was

Fig. 3. Western blot analysis of *TgA1*. Total cell lysates containing 20 µg of protein from tachyzoites (RH strain, lanes 1 and 4; ME49 strain, lanes 2 and 5) and bradyzoites (ME49 strain, lanes 3 and 6) and 3 µg of purified recombinant His₆-TgA1 fusion protein (lane 7) were subjected to SDS-PAGE on 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with antibodies prepared as described in Materials and methods (lanes 4–7) or with pre-immune serum (lanes 1–3).

Fig. 4. Suppression of the Ca²⁺ hypersensitivity of the *S. cerevisiae* *vcx1 pmc1* mutant by *T. gondii* *TgA1*. *Saccharomyces cerevisiae* *vcx1 pmc1* strain K665 was transformed with a control vector (K665pYES2) or a vector containing the entire ORF of *T. gondii* *TgA1* (K665pYES2-*TgA1*). Strain K661 has the *PMCL* gene and thus served as the positive control. The cultures were streaked on YPD (1% Difco extract, 2% Bacto-Peptone, 2% dextrose pH 5.5) plates containing 200 mM CaCl₂ (A), or were inoculated into YPD pH 5.5 with 0, 50, 100, 200 and 400 mM CaCl₂, and growth was estimated by measuring the optical density at 600 nm (B), to identify Ca²⁺-tolerant transformants.

detected in tachyzoite lysates (T). No reaction was detected when pre-immune serum was used (lower panel). Co-localization studies with the antibody against the V-H⁺-PPase and an antibody against the dense granule marker GRA2 (Labryere *et al.*, 1999) were also performed to rule out that the organelles identified as

acidocalcisomes were the dense granules. Figure 7D shows labeling of dense granules (DG) with antibodies against GRA2 (10 nm gold particles) and labeling of acidocalcisomes (V) and the plasma membrane with antibodies against the V-H⁺-PPase (arrowheads, 15 nm gold particles).

Presence of electron-dense vacuoles in *T.gondii*

Acidocalcisomes have been identified in *T.cruzi* (Scott *et al.*, 1997), *Leishmania donovani* (Rodrigues *et al.*, 1999a) and *Trypanosoma brucei* (Rodrigues *et al.*, 1999b) as the electron-dense vacuoles present in unstained parasites. We have found that similar electron-dense vacuoles are present in unstained, unfixed preparations of *T.gondii* tachyzoites (Figure 8). The contrast of a given structure in these images arises solely from its mass density since these preparations were not stained. Approximately 10–12 spherical electron-dense vacuoles of varying sizes were observed in each tachyzoite by transmission electron microscopy (Figure 8A–C). They were distributed at random over the tachyzoite's cytoplasm or occasionally arranged in rows and towards the periphery of the cells (Figure 8B, arrowheads). Groups of them were observed in some cells (Figure 8C, arrowheads). These electron-dense vacuoles do not correspond to the dense granules detected in fixed, stained preparations. X-ray microanalysis of the electron-dense organelles (Figure 9A) yielded spectra characteristic of acidocalcisomes (Docampo and Moreno, 1999). The spectrum shown is the one that yielded the most counts in 100 s (out of 14 spectra obtained), but all the other spectra taken from dense organelles were qualitatively similar: counts for phosphorus were ~2.5-fold greater than counts for calcium, which were ~3.5-fold greater than counts for magnesium. Sodium was also detected in comparable amounts to magnesium. Zinc was detectable as a trace in only a few spectra. These peaks were not present in a spectrum taken from the background (Figure 9B). Sulfur was not detected in the electron-dense vacuoles, indicating that they do not contain much

cysteine or methionine or proteins containing these amino acids. This is also in contrast to dense granules that are known to contain several proteins (Carey *et al.*, 2000).

Fig. 5. Immunofluorescence microscopy showing the localization of TgA1 in tachyzoites (A and B) and bradyzoites (E and F) of *T.gondii*. (C) Tachyzoites incubated with pre-immune serum (1:100). (D, G and H) Tachyzoites (D) and bradyzoites (G and H) incubated with monoclonal antibody against BAG-5 (1:1000). (B), (E) and (G) show the same cells as in (A), (F) and (H), respectively, by bright-field microscopy. Bar, 10 μ m.

Fig. 6. Confocal laser scanning microscopy analysis showing the localization of the Ca²⁺-ATPase (green in B, D and E) and BAG-5 (red in C, D and F) in bradyzoites (A–D) and tachyzoites (E and F) of *T.gondii* ME49 strain. (D) An overlay of (B) and (C) with no evidence of co-localization. Bar, 10 μ m.

Fig. 7. Immunocytochemical localization of Ca²⁺-ATPase (A–C), GRA2 (D) and V-H⁺-PPase (A–D) in tachyzoites of *T. gondii*, and western blot analysis of the V-H⁺-PPase (E). Note that 20 (A and D) and 15 (B and C) nm particles were used to localize the V-H⁺-PPase (arrowheads) and 10 nm particles were used for the Ca²⁺-ATPase (arrows, B and C), or GRA2 (D). V, vacuole; DG, dense granule. Bars, 500 (A), 100 (B), 120 (C) and 220 nm (D). (E) Total cell lysates containing 20 μg of protein from *T. cruzi* epimastigotes (Ep) and tachyzoites (RH strain, lane T) were subjected to SDS–PAGE on 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and probed with the antibody prepared as described in Materials and methods.

Fig. 8. Transmission electron microscopy of whole tachyzoites (A–C). Whole, unfixed and unstained cells were suspended in 0.25 M sucrose. Drops were applied to Formvar-coated grids, cells were allowed to adhere for 10 min, and then carefully blotted dry and observed directly with the Hitachi-600 electron microscope. Note the numerous electron-dense vacuoles of different sizes. Bar, 1 μm.

Discussion

In this work, we have demonstrated that *TgA1*, a gene encoding a functional Ca²⁺-ATPase, is present in the *T. gondii* genome. Comparison of the sequence of *TgA1* from *T. gondii* with other P-type ATPases indicates that this ATPase gene is closely related to the family of PMCA

Fig. 9. X-ray microanalysis of the electron-dense organelles. (A and B) The X-ray spectra recorded from an electron-dense vacuole (A) or the background (B) in whole tachyzoites.

pumps. The expression of *TgA1* in a yeast mutant deficient in vacuolar Ca²⁺ accumulation (K665) provides genetic evidence that *TgA1* encodes a vacuolar Ca²⁺ pump. This calcium pump was shown to be localized to acidocalcisomes and the plasma membrane of *T. gondii*, as indicated by immunofluorescence (Figures 5 and 6) and immunoelectron microscopy (Figure 7). This pump apparently lacks the calmodulin-binding domain present in other PMCA pumps (Strehler, 1991). This characteristic places this enzyme in a novel category together with the acidocalcisomal Ca²⁺-ATPase described in *T. cruzi* (Lu *et al.*, 1998), and the vacuolar Ca²⁺-ATPases of *S. cerevisiae* (Cunningham and Fink, 1994), *D. discoideum* (Moniak *et al.*, 1995) and *E. histolytica* (Ghosh *et al.*, 2000). As in the case of these other Ca²⁺-ATPases, we cannot rule out the possibility that calmodulin regulates *TgA1* activity by interacting with as yet unidentified sequences on the enzyme.

We also report, for the first time, the localization of the V-H⁺-PPase to the acidocalcisomes of *T. gondii*. A previous study (Rodrigues *et al.*, 2000) reported a vacuolar and plasma membrane localization of this enzyme but the vacuoles were not identified at the electron microscope level. The co-localization of a proton pump and a calcium pump to the same vacuoles is consistent with their characteristic acidity and their high calcium content. As occurs with acidocalcisomes in trypanosomatids (Docampo and Moreno, 1999) and volutin or metachromatic granules in a number of microorganisms (Meyer, 1904; Wilkinson and Duguid, 1960; Harold, 1966;

Jacobson *et al.*, 1982; Kornberg, 1995), these intracellular vacuoles appear empty with conventional electron microscopy but have a high electron density when cells are observed directly without fixation and dehydration. This has been attributed to the presence of polyphosphates bound to different cations (Wilkinson and Duguid, 1960). In this regard, we previously reported (Rodrigues *et al.*, 2000) that *T. gondii* possesses a high concentration of short chain polyphosphates. Acidocalcisomes in trypanosomatids (Urbina *et al.*, 1999) as well as volutin granules in a number of microorganisms (Harold, 1966; Jacobson *et al.*, 1982; Kornberg, 1995) have been demonstrated to contain large amounts of polyphosphates. The biochemical demonstration of acidocalcisomes in *T. gondii* means that these organelles are present in at least two evolutionarily distinct unicellular eukaryotic groups (Apicomplexa and Kinetoplastida). The presence of homologous Ca^{2+} -ATPases (Moniakakis *et al.*, 1995) and/or vacuolar H^{+} -PPases (MacDonald and Weeks, 1988; Robinson *et al.*, 1998) in other groups known to contain volutin granules, such as the slime mold *D. discoideum* (Schalterer *et al.*, 1994) and the algae *Chlamydomonas reinhardtii* (Komine *et al.*, 2000), suggests an even broader distribution and a more general biological relevance of this organelle.

In conclusion, this study provides evidence that acidocalcisomes of *T. gondii* are similar to acidocalcisomes present in trypanosomatids regarding their chemical composition, their high electron density and their content of calcium and proton pumps. Their chemical composition is also similar to the chemical composition of volutin granules in other microorganisms (Meyer, 1904; Wilkinson and Duguid, 1960; Harold, 1966; Jacobson *et al.*, 1982; Kornberg, 1995). Although volutin granules were first described almost 100 years ago (Meyer, 1904), they have not previously been investigated concerning the presence of proton or calcium pumps in their limiting membrane. This is despite the fact that they were known to be acidic and to contain large amounts of calcium (Kornberg, 1995). The presence of these organelles in many microorganisms such as bacteria, fungi, algae and protozoa, and their apparent absence in mammalian cells, makes them promising targets for chemotherapy.

Materials and methods

Culture methods

Tachyzoites of *T. gondii* RH strain were cultivated and purified according to Moreno and Zhong (1996). Tachyzoites of *T. gondii* ME49 strain were obtained from Dr Louis M. Weiss, Department of Pathology, Albert Einstein College of Medicine, New York, NY. These tachyzoites were cultivated in human fibroblasts in Dulbecco's minimum Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 100 U of penicillin/ml, 100 µg of streptomycin/ml and 0.1 µg of Fungizone/ml. After collection, they were incubated at 37°C in DMEM at pH 8.1 for 4 h (Weiss *et al.*, 1995, 1996). A 75 cm² flask with a confluent human fibroblast cell monolayer in 10 ml of DMEM at pH 7.1 was then infected with 3×10^6 tachyzoites to a final ratio of 1:3 (host:parasite). The medium was replaced on days 2 and 4 to eliminate any parasite in the supernatant. This prevented any emerging parasites from infecting more cells, leaving tissue cysts to develop further undisturbed. On days 4 and 5, bradyzoites emerged from the tissue cysts and were collected from the supernatant.

Yeast strains K665 *MATa* (*vcx1::hisGpmc1::TRP1*) and K661 *MATa* (*vcx1::hisG*), kindly provided by Dr Kyle W. Cunningham, Department of Biology, The Johns Hopkins University, Baltimore, MD (Cunningham and Fink, 1996), were grown at 30°C in standard YPD medium (1% Difco

yeast extract, Bacto Peptone, 2% dextrose) or in YPD medium pH 5.5 (adjusted with succinic acid), supplemented with 0, 50, 100, 200 and 400 mM CaCl_2 . Cell growth was assessed by measuring the optical density of the liquid cultures at 600 nm or by counting the number of colonies in plates. Both strains are isogenic and harbor the following additional mutations: *ade2-1*, *can1-100*, *his3-11,15* *leu2-3,112* *trp1-1* and *ura3-1*.

Chemicals and reagents

Fetal bovine serum, DMEM, Dulbecco's phosphate-buffered saline (PBS), Tween 20, Triton X-100, proteinase K, RNase A, HEPES, CNBr-activated Sepharose 4B, EGTA and poly-L-lysine (mol. wt 70 000) were purchased from Sigma Chemical Co. (St Louis, MO). Trizol reagent, *Taq* polymerase, SuperScript PCR buffer and Superscript II reverse transcriptase were from Gibco BRL, Life Technology Inc. (Gaithersburg, MD). The Poly(A)Tract mRNA isolation system, lambda EMBL3 phage, restriction enzymes, pGEM-T vector, Riboprobe *in vitro* Transcription System, and Prime-a-Gene Labeling System were from Promega (Madison, WI). Sequenase was from United States Biochemical Corporation (Cleveland, OH). [α -³²P]dCTP (3000 Ci/mmol), [α -³²P]UTP (3000 Ci/mmol) and the enhanced chemiluminescence (ECL) detection kit were from Amersham Life Sciences, Inc. (Arlington Heights, IL). pCR 2.1-TOPO cloning kit was from Invitrogen (Carlsbad, CA). Zeta Probe GT nylon membranes and the protein assay were from Bio-Rad (Hercules, CA). The primers were purchased from Genosys Biotechnologies Inc. (Woodlands, TX). The pET-28a⁺ expression vector, the *Escherichia coli* DE3 strain, the Quick 900 cartridge and the His-bind buffer kit were from Novagen (Madison, WI). The Protease Inhibitor Mixture Set III was from Calbiochem (La Jolla, CA). The pYES2 vector was from Stratagene (La Jolla, CA). Gold-labeled goat anti-rabbit or mouse antibodies were obtained from Ted Pella, Inc. (Redding, CA). Fluorescein and rhodamine-labeled antibodies were from Molecular Probes Inc. (Eugene, OR). The polyclonal antibody against GRA2 (Labryere *et al.*, 1999) was kindly provided by L.D. Sibley, Washington University, St Louis. All other reagents were analytical grade.

Nucleic acid analysis

DNA was isolated by standard procedures (Sambrook *et al.*, 1989). Total RNA was isolated with Trizol reagent following the manufacturer's recommendations. The polyadenylated RNA was obtained using the Poly(A)Tract mRNA isolation system. DNA was run in 1.0% agarose gels with TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0) buffer and transferred to Zeta Probe GT nylon membranes. RNA was electrophoresed in 1.0% agarose gels with 2.2 M formaldehyde, 20 mM Mops pH 7.0, 8 mM sodium acetate, 1 mM EDTA, and transferred to Zeta Probe GT nylon membranes. DNA probes were prepared using random hexanucleotide primers, Klenow fragment of DNA polymerase I (Prime-a-Gene Labeling System) and [α -³²P]dCTP. RNA probes were prepared from linearized double-stranded DNA templates with either T3 or T7 promoter sequences upstream of the probe sequence using T3 or T7 RNA polymerase (Riboprobe *in vitro* Transcription System). The hybridized filters were washed under high-stringency conditions (0.1% standard saline citrate–0.1% SDS at 65°C), unless otherwise indicated.

Oligonucleotide primers were designed to recognize the ATP phosphorylation site and the ATP binding site of cationic ATPase genes (Allen and Green, 1976; Pick and Bassilian, 1981), i.e. 5'-CGGGATCCGTNATNTGYWSNGAYAA-3' and 5'-CGGAATTCG-SRTCTTTNRYNCCR-3' as the 5' and 3' primer, respectively. PCR was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA) at 94°C for 1 min, 55–72°C for 2 min, and 72°C for 2 min/cycle (30 cycles) using *Taq* polymerase. PCR products were cloned into the pGEM-T or the pCR 2.1-TOPO vector according to the manufacturer's instructions. The cloned PCR products were sequenced and the deduced amino acid sequences were compared with the database in DDBJ/EMBL/GenBank. An ~1.4 kb PCR clone with identity to organelle-type Ca^{2+} -ATPases was used to screen genomic and cDNA libraries of *T. gondii*. The genomic library was constructed in lambda EMBL3 phage using 7–23 kb *EcoRI* fragments of genomic DNA from *T. gondii* following the manufacturer's instructions. The cDNA library was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Cat. No. 1896). Screening, selection and *in vitro* rescue of pBluescript phagemid^R from plaque-purified phages by co-infection with ExassitTM helper phage were carried out according to the manufacturer's instructions. DNA sequencing was performed by the dideoxynucleotide chain termination method of Sanger *et al.* (1977) either manually or with a 373A DNA Automatic Sequencer (Perkin Elmer Applied Biosystems, Foster City, CA). Internal

oligonucleotide primers were designed to complete the DNA sequence in both directions. DNA and deduced amino acid sequence analyses were performed using the University of Wisconsin Genetics Computer Group software package (GCG program, version 8.0). Hydrophathy analysis was done using the method of Kyte and Doolittle (1982). The α -tubulin (*TUB1*) fragment used as a control in northern blotting was obtained by amplifying *T.gondii* genomic DNA by the PCR technique, using primers TUB1 (5'-CGTCGTCGATGAGGTTTCGCA-3', 1266–1285 nt) and TUB2 (5'-TTGTGGTCCATGCGACTGAA-3', 2209–2228 nt), and the product was purified and cloned into pCR 2.1-TOPO. Densitometric analysis of northern blots was done using an ISI-1000 Digital Imaging System (Alpha Inotech Corp.). Comparison of levels of *TgAl* transcripts in tachyzoites and bradyzoites was done taking as a reference the densitometric values obtained with the *TUB1* transcripts and assuming a similar level of expression of this gene in both stages. Similar results were obtained when the densitometric values were compared by taking into account the amount of RNA added to each lane in four different experiments.

Reverse transcriptase-PCR

First strand cDNA synthesis was primed with an oligonucleotide that annealed to 710 bp downstream of the putative start codon of the *TgAl* ORF (RTP3'-1, 5'-AGTTGGAACACGGAGATCTG-3', 3797–3816 nt) in a reaction containing 1 mM dNTPs, 2.5 mM MgCl₂, 10 mM dithiothreitol, 1× SuperScript PCR buffer and 200 U of SuperScript II reverse transcriptase. Target sequences were amplified in a standard PCR using the first strand cDNA as template and primers RTP5'-1 (5'-TGAGGCTCCGGACTCCGAAG-3', 2885–2904 nt) and RTP5'-2 (5'-CGACGACGAAGACAAAGGAAG-3', 2998–3017 nt), which annealed to 223 and 109 bp upstream of the initiation codon of *TgAl* ORF, respectively, and a downstream primer that annealed to a sequence just 391 bp upstream of the primer used for first strand cDNA synthesis (RTP3'-2, 5'-TAATCCACGACTCTGACTC-3', 3406–3425 nt). PCR conditions were the same as described above except that the annealing temperature was 60°C. The product of the amplification reaction was ligated into vector pCR2.1TOPO for sequence analysis. The sequence of *TgAl* was deposited in DDBJ/EMBL/GenBank under the accession Nos AF151371 (cDNA) and AF151372 (gDNA).

Preparation of antibodies

Two primers, EXP1 (5'-CGGATCCGACGAAGTCGAGCTGATT-3') and EXP2 (5'-GAAGCTTTCAATACTTGTCTCGGCTG-3') derived from the sequences ⁶⁹⁴DEVEL⁶⁹⁹ and ⁹⁴³QPSDKY⁹⁴⁸, encoding a region of 255 amino acids within the large cytosolic loop, were used to amplify a 765 bp fragment of the *TgAl* gene. The excised fragment was ligated to the pET-28a⁺ expression vector at the *Bam*HI and *Hind*III sites, resulting in a construct that encoded the protein fused to a His₆ tag that allowed its purification on nickel-agarose columns. This plasmid was checked by DNA sequencing to ensure that the correct construct had been obtained. The recombinant plasmid was transfected into the DE3 strain of *E.coli*, the fusion protein was induced, and the expressed protein of ~33 kDa, present in inclusion bodies, was solubilized and purified according to the manufacturer's instructions. Rabbits were injected subcutaneously with 100 µg of fusion protein emulsified in Freund's complete adjuvant, followed 2 weeks later by subcutaneous injection of 100 µg of fusion protein in Freund's incomplete adjuvant. At 6, 8 and 10 weeks following the initial injection, rabbits were boosted with 100 µg of fusion protein in PBS containing a 10 mg/ml suspension of Al(OH)₃. Serum was collected before the initial injection (pre-immune serum) and 10 days after each boost. The antiserum was aliquoted and stored at -80°C. Affinity purification of anti-*TgAl* antibodies was carried out by elution from a column to which the His₆-*TgAl* fusion protein had been coupled. The affinity column was prepared by conjugating purified His₆-tagged *TgAl* (derived from a 1 l culture of the transformed *E.coli*) to CNBr-activated 4B (0.5 ml of beads) as described by the manufacturer. The affinity matrix was incubated with 10 ml of anti-*TgAl* antiserum 1:2 dilution in PBS) for 16 h at 4°C, washed four times with 20 vols of PBS, and the antibodies eluted in 0.2 M glycine pH 2.8, 1 mM EDTA. The antibodies were immediately neutralized with 0.1 vol. of 1 M Tris pH 9.5 supplemented with sodium azide to a final concentration of 0.05% and stored at 4°C. Polyclonal antiserum from Balb/c mice was raised against a keyhole limpet hemocyanin (KLH)-conjugated synthetic peptide corresponding to the *T.cruzi* V-H⁺-PPase (NH₂-⁷³⁴CNTGAWDNAKKYIEKGLRDK-KGKGS⁷⁶¹-NH₂) at the University of Illinois Biotechnology Center. *Trypanosoma cruzi* lysates used as control were prepared as described (Scott *et al.*, 1998).

SDS electrophoresis and preparation of western blots

The electrophoretic system used was essentially the same as that described by Laemmli (1970). *Trypanosoma gondii* tachyzoites and bradyzoites (1 × 10⁹) were centrifuged at 1000 g for 10 min, and resuspended in 300 µl of Dulbecco's PBS containing proteinase inhibitors (1 µg of aprotinin per ml, 1 µg of leupeptin per ml, 1 µg of pepstatin per ml and 1 mM phenylmethylsulfonyl fluoride). Aliquots of *T.gondii* (10 µl; ~20 µg of protein) were mixed with 10 µl of 125 mM Tris-HCl pH 7.5, 10% (w/v) β-mercaptoethanol, 20% (v/v) glycerol, 6.0% (w/v) SDS and 0.4% (w/v) bromophenol blue as tracking dye, and boiled for 5 min prior to application to 10% SDS-polyacrylamide gels. Electrophoresed proteins were transferred to nitrocellulose by the method of Towbin *et al.* (1979), with a Bio-Rad (Richmond, CA) transblot apparatus. Following transfer, the nitrocellulose was blocked in 5% non-fat dry milk in TPBS (0.1% Tween 20, 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl pH 7.5) overnight at 4°C. A 1:10 000 dilution of polyclonal antiserum in TPBS was then applied at room temperature for 60 min. The nitrocellulose was washed three times for 15 min each with TPBS. After incubating with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:20 000) and washing three times for 15 min each with TPBS, immunoblots were visualized on blue-sensitive X-ray film (Midwest Scientific, St Louis, MO) using the ECL chemiluminescence detection kit and following the instructions of the manufacturer.

Functional complementation of the *PMC1* gene of *S.cerevisiae* with *TgAl*

We transformed the *S.cerevisiae* *vcx1 pmc1* strain K665 with the yeast expression vectors pYES2 and pYES2-*TgAl* by a standard lithium acetate transformation procedure (Gietz *et al.*, 1992). The Ura⁺ transformants were selected by plating on synthetic-complete Ura medium (Gietz *et al.*, 1992). The *TgAl* coding region was amplified by the PCR technique from a positive cDNA λ c7 clone containing the complete *TgAl* gene and *Kpn*I and *Xba*I sites created on the PCR primers (YESP1, 5'-CGGTAC-CATGGGCGGCGTTGGGCA-3'; YESP2, 5'-CGTCTAGATCATTGC-ATTCTATGCA-3'). The *TgAl* coding region was placed at the *Kpn*I and *Xba*I sites of pYES2 with the same orientation as the *GAL1* promoter. The cultures were grown in YPD medium pH 5.5 containing 0, 50, 100, 200 and 400 mM CaCl₂ to identify Ca²⁺-tolerant transformants.

Immunofluorescence microscopy

Parasites fixed with 4% formaldehyde were allowed to adhere to poly-L-lysine-coated coverslips, permeabilized with 0.3% Triton X-100 for 20 min, blocked with 3% bovine serum albumin in PBS, and prepared for immunofluorescence with a 1:100 dilution of the antibody (anti-*TgAl*) against the 33 kDa expressed protein or a 1:1000 dilution of the monoclonal antibody against the bradyzoite surface antigen 5 (BAG-5), and a rhodamine- or fluorescein isothiocyanate-coupled goat anti-mouse or -rabbit immunoglobulin G (IgG) secondary antibody (1:160), respectively. Control preparations were incubated with pre-immune serum (1:100) or without the primary antibody. Immunofluorescence images were obtained with an Olympus BX-60 fluorescence microscope. The images were collected with a system consisting of a charge-coupled device camera (model CH250; Photometrics Ltd, Tucson, AZ), an electronic unit (model CE 200A, equipped with a 50 Hz 16-bit A/D converter) and a controller board (model NU 200; both from Photometrics Ltd). Images were acquired and evaluated by a software package (Adobe Photoshop) on a Macintosh Quadra 840 AV computer (Apple Computer, Inc., Cupertino, CA). In experiments aiming at the co-localization of Ca²⁺-ATPases and BAG-5, the samples were examined in a Leica TCS SP spectral confocal microscope. Optical sections of 0.1 µm were used.

Electron microscopy and X-ray microanalysis

For immunocytochemistry, the tachyzoites were fixed for 60 min at 4°C in a solution containing 0.1% glutaraldehyde, 4% freshly prepared formaldehyde, 1% picric acid and 3.5% sucrose in 0.1 M cacodylate buffer pH 7.2. Fixed cells were washed in PBS pH 7.2, dehydrated at -20°C in an ethanol series, and infiltrated at the same temperature in Unicryl (Scala *et al.*, 1992). Polymerization was carried out for 72 h at -20°C by UV irradiation. Thin sections were collected on 300 mesh nickel grids and blocked for 30 min with PBS containing 0.1% Tween 20 and 0.5% cold fish gelatin (PBS-TW-FG). Grids were first incubated with PBS-TW-FG containing a mouse polyclonal antiserum against V-H⁺-PPase (1:50), and a rabbit polyclonal antiserum against Ca²⁺-ATPase (1:20) or a rabbit polyclonal antiserum against GRA2 (1:150) for 120 min. After washing in PBS-TW-FG, grids were then incubated for 45 min with PBS-TW-FG containing a goat anti-mouse IgG labeled with 15 (1:75) or 20 nm (1:25) gold particles, and a goat anti-rabbit IgG (1:100) labeled

with 10 nm gold particles. Finally, grids were incubated for 15–20 min in PBS–2.5 M NaCl, washed in distilled water, stained with uranyl acetate and lead citrate, and observed in the transmission electron microscope.

For imaging whole tachyzoites, these were resuspended and washed once in 0.25 M sucrose. Drops were applied to Formvar-coated grids, cells were allowed to adhere for 10 min, and then carefully blotted dry and observed directly with the Hitachi H-600 transmission electron microscope at an accelerating voltage of 75 kV. For energy dispersive X-ray analysis, specimen grids were examined in a Hitachi H-7100FA transmission electron microscope at a voltage of 50 kV. Fine probe sizes were adjusted to cover the organelle in question and X-rays were collected for 100 s utilizing a thin window (Norvar®) detector. Analysis was performed using a Noran Voyager III analyzer with a standardless analysis identification program.

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