A Functional Aquaporin Co-Localizes with the Vacuolar Proton Pyrophosphatase to Acidocalcisomes and the Contractile Vacuole Complex of Trypanosoma cruzi*

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We cloned an aquaporin gene from Trypanosoma cruzi (TcAQP) that encodes a protein of 231 amino acids, which is highly hydrophobic. The protein has six putative transmembrane domains and the two signature motifs asparagine-proline-alanine (NPA) which have been shown, in other aquaporins, to be involved in the formation of an aqueous channel spanning the bilayer. TcAQP was sensitive to endo H treatment, suggesting that the protein is N-glycosylated. Oocytes of Xenopus laevis expressing TcAQP swelled under hypotonic conditions indicating water permeability, which was abolished after preincubating oocytes with very low concentrations of the AQP inhibitors HgCl₂ and AgNO₃. No glycerol transport was detected. Immunofluorescence microscopy of T. cruzi expressing GFP-TcAQP showed co-localization of TcAQP with the vacuolar proton pyrophosphatase (V-H²⁻-PPase), a marker of acidocalcisomes. This localization was confirmed by Western blotting and immunofluorescence staining using polyclonal antibodies against a C-terminal peptide of TcAQP. This localization was confirmed by Western blotting and immunofluorescence staining using polyclonal antibodies against a C-terminal peptide of TcAQP. In addition, there was a strong anterior labeling in a vacuole, close to the flagellar pocket, that was distinct from the acidocalcisomes and that was identified by immunogold electron microscopy as the contractile vacuole complex. Taking together, the presence of an aquaporin in acidocalcisomes and the contractile vacuole complex of T. cruzi, provides support for the role of these organelles in osmotic adaptations of these parasites.

Trypanosoma cruzi, the etiologic agent of Chagas’ disease or American trypanosomiasis, has been found to possess an acidic calcium-storage organelle that was named the acidocalcisome (1). Acidocalcisomes are also found in a diverse range of trypanosomatid and apicomplexan parasites (2), in the green algae Chlamydomonas reinhardtii (3), in the slime mold Dictyostelium discoideum (4), and in the bacterium Agrobacterium tumefaciens (5). They are characterized, in addition to their acidic nature, by their high density (both in weight and by electron microscopy), and high content of pyrophosphate (PPi), polyphosphate (polyp), calcium, magnesium, and other elements. These organelles share several properties with the vacuoles of plants (tonoplasts), such as the presence of two proton pumps, a vacuolar-type H⁻⁻⁻-ATPase and a vacuolar proton translocating pyrophosphatase (V-H²⁻⁻⁻-PPase), and a vacuolar Ca²⁺-ATPase.

One of the potential functions of acidocalcisomes is in osmoregulation (2). A link between acidocalcisomes and osmoregulation was evidenced by the rapid hydrolysis or synthesis of acidocalcisome polyP when epimastigotes of T. cruzi were submitted to hypotonic or hypertonic stress, respectively (6). A role for acidocalcisomes in the response of Leishmania major promastigotes to osmotic stress has also been proposed on the basis of their changes in sodium and chloride content after hypotonic stress (7). A functional link between acidocalcisomes and the contractile vacuole complex of C. reinhardtii (3) and D. discoideum (4), which is involved in water extrusion under hypotonic stress, has also been proposed. In this regard, early observations (8) of epimastigotes of T. cruzi, through phase contrast microscopy, have detected the presence of a contractile vacuole complex as a group of small vacuoles, which fuse as they fill. The pulsation period was found to be between 1 min and 1 min and 15 s (8). Electron micrographs of the contractile vacuole and surrounding spongiosis of other trypanosomatids have also been published (9, 10).

Aquaporins (AQPs),¹ or water channels, are important molecules for osmoregulation in a number of cells. They were initially suspected by noting that a number of cell types are much more permeable to water than predicted by simple diffusion of water through the lipid bilayer (11, 12). Aquaporins are composed of two groups; one is permeable only to water (orthodox aquaporins) and the second is permeable to water, glycerol, and other small, uncharged molecules (aquaglyceroporins) (12). At least three mammalian aquaporins (13–15) and two recently cloned aquaporins from Plasmodium falciparum (16) and Toxoplasma gondii (17), respectively, are aquaglyceroporins. Aquaporins are also abundant in the tonoplast and are also known as tonoplast intrinsic proteins (TIPs) (18, 19). A high water permeability for the tonoplast, facilitated by TIPs, is important for osmoregulation in plants (19).

In the present study, we report the cloning and sequencing of a gene from T. cruzi (TcAQP) which encodes a protein with

¹ The abbreviations used are: AQPs, aquaporins; TIPs, tonoplast intrinsic proteins; GFP, green fluorescent protein; TcAQP, Trypanosoma cruzi aquaporin, V-H²⁻⁻⁻-PPase, vacuolar proton pyrophosphatase; V-H⁻⁻⁻-ATPase, vacuolar proton ATPase; TRITC, tetramethylrhodamine isothiocyanate; MOPS, 4-morpholinoethanesulfonic acid; PBS, phosphate-buffered saline; Endo H, endoglycosidase H.
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homology to plant aquaporins. The water transport properties of Tc-AQP were analyzed by expressing the gene in Xenopus laevis oocytes and performing oocyte swelling assays. TcAQP was shown to localize to the acidocalcisomes and to the contractile vacuole complex of the parasites. Together, these results provide evidence for the presence of a functional aquaporin in acidocalcisomes and contractile vacuoles, and support a role for these organelles in osmoregulation.

EXPERIMENTAL PROCEDURES

Culture Methods—Different stages of T. cruzi (Y strain) were obtained as described previously (20).

Chemicals and Reagents—Fetal bovine serum, Dulbecco's phosphate-buffered saline (PBS), peroxidase labeled concanavalin A, and protease inhibitor mixture were purchased from Sigma. Restriction enzymes, T4 DNA ligase, Taq polymerase, the Klenow fragment of DNA polymerase, SuperScript PCR buffer, and SuperScript II reverse transcriptase, TRIZol reagent, and the enhanced chemiluminescence (ECL™) detection kit were obtained from Amersham Biosciences. Alexa 488 and Alexa 546-labeled antibodies were from Molecular Probes Inc. (Eugene, OR). mMESSAGE mACHINE™ transcription kit was from Ambion (Austin, TX). The pSP64T vector was a gift from David Miller (University of Illinois at Urbana-Champaign). Oligonucleotides were synthesized at Integrated DNA Technologies, Inc. (Coralville, IA). TRITC-concanavalin A conjugate was from Molecular Probes (Eugene, OR). All other reagents were analytical grade.

Isolation of the T. cruzi AQP gene and DNA Sequencing—To screen for genes encoding AQP in T. cruzi, the amino acid sequence of human AQP-1 (NM_000385) was used to search the Integrated T. cruzi Genome Resource using tBLASTn. This search yielded five sequences and the expressed sequence tag clone 24g3 (5). A polyadenylated RNA was obtained with the PolyATtract mRNA isolation system. RNA samples were subjected to electrophoresis in 1% agarose gel, and the proteins were transferred to nitrocellulose and the nitrocellulose was blocked in 5% non-fat dry milk in Tween-PBS (0.1% Tween 20, 80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, pH 7.5) overnight at 4 °C. A 1.2000 dilution of purified polyclonal antiserum in 3% bovine serum albumin in Tween-PBS was then applied at room temperature for 60 min. The nitrocellulose was washed three times for 15 min each with Tween-PBS. After 30 min of incubating with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2000) and washing three times for 15 min each with Tween-PBS, immunoblots were visualized on blue-sensitive x-ray film using the ECL™ chemiluminescence detection kit and following the instructions of the manufacturer (Amersham Biosciences). The TcAQP gene was amplified by PCR using Tc endoglycosidase H and TRITC-concanavalin A Treatment—Acidocalcisome fractions obtained as described previously (24) were incubated in the absence or presence of endoglycosidase H according to the manufacturer’s instructions. Control reactions containing 15 µg of ovalbumin confirmed activity of the enzymes. After the incubation, the samples were precipitated for 2 h at 4 °C with 750 µl of ethanol and pelleted at 16,000 × g for 30 min at 4 °C. The dried pellets were resuspended in sample buffer and boiled for 5 min before SDS-polyacrylamide gel electrophoresis (20%). The proteins were transferred from the gels to nitrocellulose and probed with anti-TcAQP polyclonal antiserum and stripped. Membranes were reprobed with peroxidase labeled concanavalin A (10 µg/ml) at room temperature for 30 min and washed three times for 15 min each with Tween-PBS. Finally, membranes were visualized on blue-sensitive x-ray film using the ECL™ chemiluminescence system.

Construction of GFP-TcAQP Expression Plasmid and Transfections—The coding region of the green fluorescent protein (GFP) was amplified from pXG-GFP+ vector by PCR using Pfu DNA polymerase with a forward oligonucleotide primer containing a HindIII site (5'-GCGGGAAGCTTATGCTAGGGAGGAGGAGGAG-3') and a reverse oligonucleotide primer with an added N-terminal cysteine (N-LDTHDRVA-C). The peptide was cross-linked by its primary amine to keyhole limpet hemocyanin and used to immunize a rabbit. Polyclonal antibodies were affinity purified using HiTrap-protein A HP columns. Samples of acidocalcisomal (1.5 µg of protein) (24) and total membranes of T. cruzi epimastigotes (1.5 or 15 µg of protein) (25) were mixed with sample buffer (125 mM Tris-HCl, pH 7, 10% v/v β-mercaptoethanol, 20% v/v glycerol, 4% w/v SDS, and 4% w/v bromophenol blue) and boiled for 5 min before application to SDS-polyacrylamide gels (12%). After electrophoresis on the gel, the nitrocellulose was blocked in 5% nonfat dry milk in Tween-PBS (0.1% Tween 20, 80 mM NaHPO4, 20 mM NaH2PO4, 100 mM NaCl, pH 7.5) overnight at 4 °C. A 1.2000 dilution of purified polyclonal antiserum in 3% bovine serum albumin in Tween-PBS was then applied at room temperature for 45 min. The nitrocellulose was washed three times for 15 min each with Tween-PBS. After 30 min of incubating with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2000) and washing three times for 15 min each with Tween-PBS, immunoblots were visualized on blue-sensitive x-ray film using the ECL™ chemiluminescence detection kit and following the instructions of the manufacturer (Amersham Biosciences).
slips, permeabilized for 5 min with PBS/0.3% Triton X-100, blocked for one hour with PBS/3% bovine serum albumin/5% goat serum/50 mM NH₄Cl, and incubated with primary antibody for 1 h and then secondary antibody for 45 min. For visualization of the vacuolar H⁺/H₁₀₀₁/pyrophosphatase, mouse polyclonal anti-\(T. cruzi\) pyrophosphatase was used at 1:200, followed by goat anti-mouse Alexa 488 or Alexa 546 at 1:1000. For visualization of aquaporin, rabbit polyclonal anti-\(T. cruzi\) aquaporin was used at 1:200, followed by goat anti-rabbit Alexa 546 at 1:1000.

For visualization of the flagellar pocket and/or cytostome, an adaptation of a previously described method was used (27). Briefly, cells were fixed in 4% formaldehyde, adhered to poly-L-lysine coverslips, and then incubated in buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 MgSO₄, 5.5 mM glucose, 50 mM Hepes, pH 7.2) containing 3% bovine serum albumin and 5 \(\mu\)M TRITC-ConA for 1 h before visualization.

Confocal images were collected with a Leica laser scanning confocal microscope (TCS SP2) using a 63\(\times\) Plan-Apo objective with NA 1.32. Single optical sections were recorded with an optimal pinhole of 293 nm according to Leica instructions.

**Electron Microscopy**—For immunocytochemistry, cells were fixed in 0.1% glutaraldehyde, 4% paraformaldehyde, 0.8% picric acid, 3.5% sucrose, 0.1 \(\times\) cacodylate, pH 7.3, embedded in unicyl, and sectioned onto Formvar-coated grids according to standard methods. Grids were blocked for 1 h in PBS/0.1% Tween 20% fish gelatin, and incubated with the primary antibody for 3 h and the secondary antibody for 1 h.

For detection of \(TcAQP\), polyclonal anti-aquaporin was used at 1:10 followed by goat anti-rabbit 10-nm gold at 1:75. For detection of \(Tc\) \(\gamma\)-P-Pase, polyclonal anti-\(Tc\) \(\gamma\)-P-Pase was used at 1:50, followed by goat anti-mouse 20-nm gold at 1:15.

**Oocyte Expression of \(TcAQP\) and Osmotic Swelling Assay**—The entire coding sequence of the \(TcAQP\) gene was amplified by PCR using PFu DNA polymerase. Oligonucleotide primers for amplification of the AQP coding region, \(TcAQP5\) (5'-GAAGATCTATGACGTTCTCTCCGGGT-3') and \(TcAQP3\) (5'-GAAGATCTTCAGAAAACCTGACCTGA-3'), were designed so that BglII restriction sites were introduced at the 5'- and 3'-ends for convenient cloning in the pSP64T oocyte expression vector, which contains the 5'- and 3'-untranslated regions of the \(\beta\)-globin gene of \(X. laevis\). Capped cRNA was transcribed in vitro using SP6 mMESSAGEMACHINE™ transcription kit after linearization of the plasmid with SmaI. Stages V and VI \(X. laevis\) oocytes were defolliculated by incubating ovarian fragments in 0.15% collagenase type II for two hours at room temperature and were injected with 32 nl of water or 5–10 ng of cRNA in 32 nl of water. Injected oocytes were maintained for 3 days at 18°C in Barth's buffer (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.41 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.4, osmolality 200 mOsm) prior to osmotic swelling test.

**Fig. 1. Sequence analysis of an aquaporin from \(T. cruzi\).** A, the deduced amino acid sequence of \(T. cruzi\) \(AQP\) (AAM76680) is compared with the AQP sequences of \(P. falciparum\) (AJ413249), human AQP1 (NM_000385), and Aqy1p from \(S. cerevisiae\) (NP_015518). Similar residues are shaded, the six membrane-spanning segments are underlined, the NPA motifs are indicated by asterisks above the alignment, and the potential N-glycosylation site is identified in a box. B, hydropathy analysis of \(TcAQP\) (Kyte-Doolittle algorithm) is consistent with six membranespanning regions (numbered 1–6) and five connecting loops (A–E). C, phylogenetic tree analysis showing the 10 known mammalian aquaporins (AQP0–9) (NP_036196, NM_000385, NP_000477, A57119, NP_001641, NP_001642, NP_001643, NP_001161, NP_001160, NP_066190) as well as the \(E. coli\) aquaporin AQPZ (AAC43518) and the glycerol facilitator glpF (NP_290556), the aquaglyceroporin from \(P. falciparum\) (AJ413249), Aqy1p (NP_015518) and Aqy2p (AAD10058) from \(S. cerevisiae\), and \(\gamma\)-TIP (NP_181221) and PIPα (CAA53475) from \(A. thaliana\). The bar indicates a branch length corresponding to 0.1 substitutions per site. Distances were calculated using the Neighbor Joining (NJ) method of Saitou and Nei (23).
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RESULTS

Isolation of the T. cruzi AQP Gene and Sequence Analysis—The complete coding region of TcAQP was established as described under “Experimental Procedures,” and the translation of the open reading frame of 693 bp yielded a polypeptide of 231 residues with a predicted molecular mass of 24,681 Da. A BLAST search of the protein data base showed that the amino acid sequence from T. cruzi AQP has 26–38% identity and 40–55% similarity to other AQPs (Fig. 1A). The T. cruzi protein contains an internal repeat characteristic of all AQPs. The N- and C-terminal halves are sequence-related and each has the signature motif Asn-Pro-Ala (NPA) that forms a single transmembrane domain (1–6) and five connecting loops (A–E). Cys189 in loop E of the AQP1 was shown to be the mercury-sensitive residue (30).

TcAQP has a glycine (Gly168) at the position equivalent to Cys189 in AQP-1, however it has two cysteines nearby (Cys183 and Cys188). There is a third cysteine at residue 121, which is conserved motive NPA, and also by AgNO3 (32). The increase in water permeability (Pf, μm/s) were determined from osmotic swelling data, initial oocyte volume (V0 = 9 × 10−4 cm3), oocyte surface area (S = 0.045 cm2), and the molecular ratio of water (Vw = 18 cm2/mol) by the following formula: Pf = V0 × dV/Vw × dt/S × Vw × (osm0 − osm∞).

Functional Expression of TcAQP in Xenopus Oocytes—The basic transport characteristics of TcAQP were analyzed by oocyte swelling assay. Fig. 3A shows the osmotic swelling curves of control oocytes and of oocytes expressing TcAQP after subjecting them to hypotonic shock in diluted Barth’s buffer (from 200 to 70 mOsm). Water-injected oocytes swelled minimally (Pf = 8.35 ± 3.2 μm/s), whereas TcAQP-injected oocytes swelled and ruptured within 5 min (Pf = 31.8 ± 9.1 μm/s). The coefficient of water permeability Pf was 3.8-fold higher than in water-injected oocytes (Fig. 3B).

Oocytes expressing the protein from T. cruzi were analyzed for glycerc permeability in an isosmotic swelling assay with 130 mS glycerol. Oocytes injected with TcAQP cRNA were not permeated by glycerol (Pf = 2.27 ± 0.01 μm/s). On the contrary, shrinkage of these cells was observed in comparison with control oocytes (Fig. 3A and B). TcAQP contains three cysteines of which one is found in a transmembrane domain, and two are part of a membrane-spanning domain (loop E). Osmotic water permeability though water channels is inhibited by HgCl2, via covalent modification of a cysteine residue in the vicinity of the conserved motive NPA, and also by AgNO3 (32). The increase in water permeability of TcAQP-expressing oocytes was inhibited by incubation in 300 μM of HgCl2 (Pf = 9.7 ± 4.5 μm/s), the inhibition being partially reversed by subsequent incubation in 5 mM β-mercaptoethanol (Pf = 20.5 ± 5.6 μm/s), as shown in Fig. 3, A and B. At low concentrations of HgCl2, ranging from 1 to 10 μM, the water permeability was also inhibited in TcAQP-injected oocytes (Fig. 3, C and D). The effect of AgNO3 was analyzed at concentrations between 1 and 10 μM. As shown in Fig. 3, E and F, AgNO3 was a potent inhibitor of TcAQP.

Localization of TcAQP in T. cruzi—To investigate the localization of TcAQP we generated fusion proteins containing the GFP at the C-terminal of TcAQP. Immunofluorescence microscopy of T. cruzi epimastigotes expressing this protein showed localization in acidocalcisomes of TcAQP-GFP (Fig. 4B) with TcPPase (Fig. 4C), a known marker of acidocalcisomes (24), as
Fig. 3. Osmotic water permeability, isosmotic glycerol permeability, and HgCl₂ and AgNO₃ inhibition of oocytes expressing TcAQP. **A,** shows the osmotic swelling of mock-injected (closed circles) and TcAQP cRNA-injected oocytes (open circles) in diluted Barth's buffer (70 mOsm). The relative volume change of cRNA-injected oocytes (closed squares) in medium with 88 mM NaCl isotonically substituted by 130 mM glycerol. Inhibition of osmotic water permeability of TcAQP cRNA injected oocytes with HgCl₂ (0.3 mM) (open triangles), and restoration with β-mercaptoethanol (closed triangles). **B,** Pᵢ values (mean ± S.D.) were calculated from the swelling curves in **A.** C, inhibition of the osmotic swelling of cRNA-injected oocytes in diluted Barth's buffer with 1 (open triangles), 5 (closed squares), and 10 μM (closed triangles) of HgCl₂. Mock-injected (closed circles) and TcAQP cRNA-injected oocytes (open circles) in Barth's buffer (70 mOsm) without HgCl₂. D, Pᵢ values (mean ± S.D.) were calculated from curves in **C.** E, inhibition of the osmotic swelling by AgNO₃. TcAQP cRNA-injected oocytes incubated in diluted Barth's buffer with 1 (open triangles), 5 (closed squares), and 10 μM (closed triangles) AgNO₃. Mock-injected (closed circles) and TcAQP cRNA-injected oocytes (open circles) in Barth's buffer (70 mOsm) without AgNO₃. F, Pᵢ values (mean ± S.D.) were calculated from curves in **E.** The number of oocytes tested is shown between parentheses.
detected with polyclonal antibodies prepared against a conserved epitope in the *T. cruzi* enzyme. In addition, there was an anterior localization of *Tc*AQP in a vacuole, close to the flagellar pocket, that was distinct from the acidocalcisomes (Fig. 4D, arrowhead). Concanavalin A has been shown to bind to the flagellar pocket of *Leishmania donovani* (27) or to the cytostome of *T. cruzi* (33). The cytostome is a specialized structure formed by invagination of the plasma membrane in a region close to the flagellar pocket of epimastigotes and amastigotes forms, and is involved in endocytosis (34). When cells transfected with *Tc*AQP-GFP (Fig. 4F) were reacted with a concanavalin A-TRITC conjugate (Fig. 4G) to label those struc-
tures (27, 33), there was no co-localization (Fig. 4H), suggesting that most TcAQP-GFP resided in a compartment close to but distinct from the flagellar pocket and/or cytostome. The same aquaporin distribution was observed in epimastigotes reacted with affinity-purified polyclonal antibody against TcAQP (Fig. 4K), while preimmune serum gave no reaction (not shown). These results also confirmed that the GFP-AQP pattern is not an artifact of protein overexpression and/or mistargeting. Labeling of acidocalcisomes with antibody against TcAQP (Fig. 4K) was more complete than with TcAQP-GFP (Fig. 4, B and F) because of the difficulties associated with high resolution imaging of GFP. However, some acidocalcisomes were labeled with antibodies against TcAQP but not with antibodies against TcPPase, and vice versa. Similar heterogeneity in labeling of acidocalcisomes with antibodies against a V-H⁺-ATPase and a Ca²⁺-ATPase has been reported before (35) and could suggest the existence of different populations of acidocalcisomes. However, we cannot rule out the possibility that some aquaporin could be labeling another unidentified organelle. In this regard, Sarkar et al. (36) recently reported a punctate distribution of phosphoenolpyruvate mutase in T. cruzi that does not co-localize with a variety of markers. Similar results to those observed with epimastigotes were obtained with infective stages. Co-localization of TcAQP-GFP (Fig. 5, B and J) or antibodies against TcAQP (Fig. 5, G and O) with antibodies against TcPPase (Fig. 5, C, F, K, and N) was detected in amastigotes (Fig. 5, D and H) and in trypomastigotes (Fig. 5, L and P). Labeling of a distinct vacuole was also detected in both stages (arrows in Fig. 5, D, H, L, and P).

Presence of a Contractile Vacuole Complex in Different Life Cycle Stages of T. cruzi, Association with Acidocalcisomes, and Labeling with Antibodies Against Aquaporin—Acidocalcisomes have been linked to the function of a contractile vacuole complex in C. reinhardtii (3) and D. discoideum (4) and the presence of a contractile vacuole complex has been reported in different trypanosomatids (10, 11), including T. cruzi (9). However, there has been only occasional mention of this structure (37) even though extensive electron microscopy studies have been done in T. cruzi. Since in trypanosomatids the contractile vacuole complex is located close to the flagellar pocket we explored the possibility that aquaporin could be located in this structure in T. cruzi.

In all organisms examined to date, the contractile vacuole complex consists of a large central bladder surrounded by a diffuse radial network of tubules and vesicles known as the spongiome (38). In all three life cycle stages of T. cruzi, a large irregular-to-round vacuole that was associated with a loose network of tubules could be easily identified by transmission electron microscopy in close opposition to the flagellar pocket (Fig. 6, A–D). These structures closely matched the descriptions of the contractile vacuole fine structure in the trypanosomatids Leptomonas collosoma (10) and Bodo sp. (11), as well as descriptions from other non-trypanosomatid organisms (39–41). Interestingly, many of the observed acidocalcisomes and con-
tractile vacuole bladders contained remnants of electron dense material (closed arrowheads). Accumulation of this material is generally diagnostic for acidocalcisomes under transmission electron microscopy (2), and suggests communication between the two compartments. Occasional observation of acidocalcisomes apparently fusing with the contractile vacuole (open arrowhead in Fig. 6B) further served to reaffirm this hypothesis.

In order to analyze in more detail the structures labeled with the TcAQP-GFP, or with antibodies against TcAQP, immunoelectron microscopy was performed on thin sections of parasites embedded in the hydrophilic resin Unicryl using the polyclonal antibody against the C-terminal of TcAQP and monoclonal antibodies against TcPPase. TcAQP and TcPPase co-localized to acidocalcisomes (Fig. 7E). TcAQP was also highly concentrated in the contractile vacuole and the immediate surround-
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Fig. 8. Immunoblot analysis with antibody against a C-terminal synthetic peptide of TcAQP. A, detection of TcAQP by immunoblot using an affinity-purified polyclonal antibody against a C-terminal peptide of the channel. Amastigote (A), trypomastigote (T), and epimastigote (E) proteins (15 μg/ lane) were separated by SDS-PAGE and transferred to nitrocellulose. B, detection of TcAQP by immunoblot in membrane and acidocalcisome fractions from epimastigotes (1.5 μg of protein/lane). C, acidocalcisomal fractions containing 1.5 μg of protein were incubated in the absence (−) or presence (+) of Endo H before immuno blotting. Panel a shows a band of lower apparent molecular mass after Endo H treatment. This band was not stained when the blot was treated with peroxidase-labeled concanavalin A (panel b).

DISCUSSION

We report here that a gene, TcAQP, present in the T. cruzi genome encodes a functional aquaporin. The open reading frame corresponding to TcAQP encodes a protein of 231 amino acids and a molecular mass of 24.7 kDa (Fig. 1A) that is expressed in all life stages of the parasite (Figs. 2B, 4, 5, and 8). The TcAQP is present as a single-copy gene (Fig. 2A) and is a member of the orthodox (water-transporting) family of aquaporins (Fig. 1C).

TcAQP was expressed in X. laevis oocytes, where it was able to function as a water, but not a glycerol channel (Fig. 3). TcAQP was found to have similar sensitivity to AgNO₃ and HgCl₂. This is in contrast to other aquaporins that are about 20 times more sensitive to AgNO₃ than to HgCl₂ (32). The mechanism of silver and mercury inhibition is most likely due to their ability to interact with sulfhydryl groups of proteins. In the case of TcAQP it is likely that these metals react with the sulfhydryl group of cysteines in the vicinity of the conserved NPA motif and thus effectively block the constriction region of the water channel. TcAQP has two cysteines in the vicinity of the NPA motif (Cys₁₈₃ and Cys₁₈₈) that could potentially react with these metals. The coefficient of water permeability (Pₒ) was enhanced by 3.8-fold by injecting mRNA of TcAQP into Xenopus oocytes. The result suggests low water channel activity of TcAQP. In this regard, it has been suggested (12) that the low water permeability exhibited by AQP0 and AQP65 may reflect the need for an activation step, and we cannot rule out that this might be the case with TcAQP.

Immunofluorescence microscopy using a fusion protein containing the green fluorescent protein at the C-terminal of TcAQP or antibodies against a C-terminal peptide of TcAQP (Figs. 4 and 5) revealed its localization in acidocalcisomes and in a vacuolar structure close to the flagellar pocket of the parasites. This vacuolar structure was not the cytosome, as demonstrated by the absence of co-localization of TcAQP with concanavalin A, a marker of the cytosome of T. cruzi (33) (Fig. 4H), and was identified by electron microscopy as the contractile vacuole complex (Figs. 6 and 7). Both TcAQP and V-H⁺-PPase co-localized to the acidocalcisomes and contractile vacuole complex. Further work will be necessary to investigate the mechanism by which dual targeting of T. cruzi aquaporin occurs.

The adaptation of a number of protozoa to hypsomotic stress involves, in addition to the release of ions and osmolytes, as occurs in mammalian cells (42), the release of water by a contractile vacuole complex. Recent work has shown that most, if not all, contractile vacuole complexes are composed of a two-compartment system enclosed by two differentiated membranes (38, 43). One membrane (spiongome), which is often divided into numerous vesicles and tubules, contains many proton-translocating V-H⁺-ATPase enzymes that provide an electrochemical gradient of protons for water transport and which can fuse only with the membrane of the second compartment. The membrane of the second compartment (bladder) which lacks V-H⁺-ATPase holoenzymes, although has a V-H⁺-PPase (3, 4) expands into a reservoir for water storage, and is capable of fusing with the plasma membrane (38, 43). It is this second compartment that periodically undergoes contraction, with the expulsion of water (38). How water is accumulated in the contractile vacuole complex was unknown. Although a water channel was postulated to be involved (38) it was never before identified. Our work provides the first evidence for the presence of an aquaporin in the contractile vacuole complex. Interestingly, other vacuoles, besides the contractile vacuole, have been observed to take up water when some protozoa are placed in hypsomotic media (44, 45), and they have been suggested to also play a role in volume homeostasis (46). It is possible that these vacuoles correspond to the acidocalcisomes that we described in several protozoa (2–4).

As T. cruzi progresses through its life cycle, it encounters diverse, severe environmental stressors to which it must successfully adapt. Of particular interest is the parasite’s ability to cope with extreme fluctuations in osmolarity that occur within the gut of the vector (47, 48) and also as the parasite moves...
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from the insect gut through the acidic phagolysosome to the cytosol of the host cell (49). The infective form of the parasite passes out of the vector in the highly concentrated excreta and rapidly encounters the interstitial fluid of the mammalian host with a much lower osmolarity. Clearly the parasite must have mechanisms that allow it to adapt both to hyperosmotic and hypotonic stress. In this work we have identified the presence of an aquaporin in acidocalcisomes and contractile vacuole complex of T. cruzi, providing support for the role of these organelles in osmotic adaptations of these parasites.

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