IDENTIFICATION OF PROTEINS LOCALIZED TO THE CONTRACTILE VACUOLE OF *TRYPANOSOMA CRUZI*

by

MIYOUNG PARK

(Under the direction of Silvia Moreno)

ABSTRACT

Trypanosoma cruzi is the etiologic agent of Chagas disease and is able to survive in a wide range of environments as it progresses through its life cycle. Fluctuations in osmolarity occur within the gut of the vector and as the parasite moves from the insect gut to the bloodstream, acidic phagolysosomes, and host cell cytosol. Thus, the parasites have mechanisms to respond to both hypo-osmotic and hyper-osmotic stresses. The contractile vacuole complex is an osmoregulatory organelle, which controls intracellular water balance by accumulating excess water and expelling it from the cell. Although recent studies showed the contractile vacuole is involved in volume regulation in *T. cruzi,* how the contractile vacuole mediates this process is poorly understood. We will identify the proteins present in the contractile vacuole in order to clarify the mechanism.

In a previous proteomic study done in the laboratory a number of proteins were identified in a fraction enriched for the contractile vacuole. To validate the proteomic data we chose to confirm the localization of proteins found in this fraction and with homology to those found also in the *Paramecium tetraurelia*, *Dictyostelium discoideum and Acanthamoeba castellanii* contractile vacuole complex.

V-H^+-ATPase, LvsA, drainin and Rab11 are known to regulate the contractile vacuole system and the pathways that control contractile vacuole discharge in *Dictyostelium*. Clathrin and AP180, major components of the cytoplasmic coat found on clathrin coated vesicles localize to the contractile vacuole and are required for the biogenesis of the contractile vacuole system in *Dictyostelium*. SNAREs are intracellular trafficking proteins that localize to the contractile vacuole and are involved in the function of the contractile vacuole in *Paramecium*.

In order to study the localization of these proteins, we cloned six contractile vacuole protein-coding genes (V-H^{+-}ATPase subunit B, V-H^{+-}ATPase subunit a, SNARE2-1, SNARE2-2, AP180, and Disgorgin/Drainin) from *T. cruzi*. To localize contractile vacuole proteins, we generated stable cells overexpressing the contractile vacuole proteins tagged with green fluorescence protein (GFP). As a result, we show that the TcV-H^+-ATPase subunit B, subunit *a*, and TcAP180 localize to the bladder of the contractile vacuole, two SNAREs, TcSNARE2.1 and TcSNARE2.2 localize to the spongiome of the contractile vacuole, and TcDisgorgin/Drainin is cytsolic and in the flagella pocket. Overall, we have demonstrated the presence of six new contractile vacuole proteins in *T. cruzi* using proteomic analysis and overexpression of the contractile vacuole proteins with GFP fusion in *T. cruzi.*

INDEX WORDS:

Tryanosoma cruzi (*T. cruzi*), contractile vacuole (CV), bladder and spongiome of the contractile vacuole, regulatory volume decrease (RVD), V-H^+-ATPase subunit B, V-H^+-APTase subunit *a*, SNARE2.1, SNARE2.2, AP180, Disgorgin/Drainin

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INTRODUCTION

1. *Trypanosoma cruzi* and regulatory volume decrease

The protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) is the etiologic agent of Chagas disease or American trypanosomiasis, the leading cause of cardiac death in Latin America (Urbina and Docampo 2003; Rohloff and Docampo 2008).

The *T. cruzi* is transmitted through the feces of triatomine insect vectors, called kissing bugs, to mammalian hosts, including rodents, sloths, armadillos, marsupials, dogs, and humans. The obligatory transmission between insect vectors and mammalian hosts is maintained in a sylvatic cycle. Transmission to the mammal via contamination of parasite-laden feces into broken skin or mucous membranes usually takes place during an insect blood meal. While an infected insect vector takes a blood meal, releases trypomastigotes are released from its feces to near the site of the bite wound. Trypomastigotes enter the host through the wound or through intact mucosal membranes. Inside the host, the trypomastigotes invade cells near the site of infection, where they differentiate into intracellular amastigotes. The amastigotes reproduce by binary fission and differentiate into trypomastigotes, and then are released into the circulation as bloodstream trypomastigotes. Trypomastigotes infect cells from a variety of tissues and transform into intracellular amastigotes in new infection sites. The bloodstream trypomastigotes do not replicate. Replication resumes only when the parasites enter another cell or are ingested by another vector. The ingested trypomastigotes transform into epimastigotes in the vector's midgut.

The parasites multiply and differentiate in the midgut and differentiate into infective metacyclic trypomastigotes in the hindgut (Andrade and Andrews 2005).

While *T. cruzi* passes through a digenetic life cycle, the parasite encounters diverse environmental fluctuations to which it acclimates in order to survive. The infective trypomastigote passes from the excreta (600-700 mOsm) of the triatomine insect vector (Kollien *et al.* 2001) and encounters the interstitial fluid of the mammalian host with a much lower osmolarity (330 mOsm) (Rohloff and Docampo 2008). Physiological responses to hypo-osmotic conditions have been particularly studied in mammalian cells. Upon hypo-osmotic stress, cells initially swell but regain nearly normal cell volume by a process termed the regulatory volume decrease (RVD) (Lang *et al.* 1998). Like mammalian cell, *T. cruzi* exhibits a strong RVD when exposed to hypo-osmotic conditions. The RVD of *T. cruzi* results from the efflux of various inorganic ions, organic osmolytes, and amino acids to the extracellular environment. This process is complete in all *T. cruzi* stages by 5 min (Rohloff *et al.* 2004; Rohloff and Docampo 2008).

2. Roles of the contractile vacuole complex and other organelles involved in response to osmotic stress

2.1 Contractile vacuoles and osmotic stress

The contractile vacuole complex is an organelle that controls the intracellular water balance by accumulating and expelling excess water from the cell. Recent work has shown that the contractile vacuole is composed of a two compartment system enclosed by two differentiated membranes (Allen and Naitoh 2002). The spongiome is divided into numerous vesicles and tubules. The spongiome can only fuse with the membrane of the second compartment and contains a V-H^+-ATPase that provides an electrochemical gradient of protons for water transport. The spongiome is a fluid storing compartment in *Paramecium* (Allen 2000) and function as collecting ducts to accumulate excess water in *Dictyostelium* (Du *et al.* 2008). The second compartment of the contractile vacuole, the bladder, expands as a reservoir for water storage (Rohloff and Docampo 2008) and periodically expels water (Allen and Naitoh 2002). The contractile vacuole bladder takes up water when protozoa are placed in hypo-osmotic media (Cronkite *et al.* 1991).

Furthermore, the contractile vacuole plays an important role in calcium homeostasis as an intracellular calcium store. Calmodulin was found to be associated with the contractile vacuole complex in *P. tetraurelia, T. pyriformis,* and *D. discoideum*. Studies in *P. multimicronucleatum* suggested that the contractile vacuole is also involved in excretion of calcium under high calcium conditions.

2.2 Acidocalcisomes and osmotic stress

Acidocalcisomes are acidic calcium-containing organelles present in eukaryotes. Those organelles are involved in RVD and change their polyphosphate (polyP) and ionic content following osmotic changes (Docampo *et al.* 2005). According to a recently proposed RVD mechanism for *T. cruzi,* an aquaporin is transferred to the contractile vacuole from acidocalcisomes under hypo-osmotic conditions (Montalvetti *et al.* 2004). Fusion of acidocalcisomes with the contractile vacuole may be induced by formation of cAMP by an adenylyl cyclase. In addition, acidocalcisomes release an acidocalcisomal exopolyphosphatase that cleaves polyphosphate and releases inorganic phosphate and various polyphosphate-chelated osmolytes as osmotically active substances. The resulting osmotic gradient results in water accumulation in the contractile vacuole through the aid of the aquaporin. The water is ejected into the flagellar pocket (Figure 1).

2.3 General roles of contractile vacuole proteins involved in the contractile vacuole complex

2.3.1 Vacuolar-H^+-ATPase

The vacuolar H^+-ATPase (V-ATPase) translocates protons across membranes generating an electrochemical gradient through ATP hydrolysis. The V-ATPase is responsible for acidification of organelles like phagosomes, lysosomes, early endosomes, the trans-Golgi network, dense core secretory granules, and vacuoles of plants and fungi (Stevens and Forgac 1997). The V-ATPase also plays an important role in the regulation of the cytosolic pH and the uptake of cations such as Na^+, Ca^{2+}, and Cd^{2+} *via* H^+-driven antiporters (Dietz *et al.* 2001).

The V-ATPases consist of two subcomplexes: V_0 and V_1. The cytosolic V_1-subcomplex binds and hydrolyzes ATP, and protons pass through the membrane *via* the V_0-subcomplex. The

V-ATPase in *S. cerevisiae* is composed of 14 different subunits. V_1 contains subunits A-H, and V_0 is formed by the subunits *a, c, c', c'', d* and *e* (Figure 2) (Ishida *et al.* 1996; Beyenbach and Wieczorek 2006) .

In *Paramecium multimicronucleatum*, V-ATPase localized on the decorated spongiomes near the radial arms of the contractile vacuole complex (Ishida *et al.* 1996). In *Dictyostelium,* the V-ATPase is found in membranes of the contractile vacuole complex and regulates the pathways that control contractile vacuole discharge (Heuser *et al.* 1993; Clarke *et al.* 2002).

V-H^+-ATPase Subunit B

Subunit B (55~60 kDa) was first cloned from *Neurospora* (Margolles-Clark *et al.* 1999) and *Arabidopsis* (Finbow and Harrison 1997). The orthologous protein of other species shows high level of identity (34 to 80%). The subunit B also shows some 25% sequence identity with V-ATPase subunit A in yeast. The subunit B has a consensus sequence for nucleotide binding (Finbow and Harrison 1997). In *T.cruzi*, V-ATPase subunit B has ~70% identity to homologous proteins in *Dictyostelium*, human, and yeast.

V-H^+-ATPase subunit *a* (100 kDa Subunit)

Subunit *a* of V-ATPase (96~116 kDa protein) has been cloned from rat brain (Perin *et al.* 1991), human (Li *et al.* 1996), yeast (Manolson *et al.* 1992), and *Dictyostelium* (Liu and Clarke 1996) . It has an N-terminal hydrophilic domain exposed to the cytoplasm and a C-terminal domain containing seven putative membrane-spanning hydrophobic segments. The subunit a may be involved in coupling ATP hydrolysis to proton translocation (Finbow and Harrison 1997). *T. cruzi* V-ATPase subunit *a* has ~40% identity to orthologues in *Dictyostelium* and human.

2.3.2 SNARE2

Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) belong to families of small and membrane-associated proteins that are distributed on the cytoplasmic surfaces of all membranes of the secretory pathway and are involved in membrane trafficking (Mayer *et al.* 1996; Weber *et al.* 1998). The membrane trafficking of transport vesicles delivers proteins, hormones, or neurotransmitters throughout the cell in all eukaryotes (Schekman and Orci 1996).

N-ethylmaleimide-sensitive factor (NSF) and soluble NSF attachment protein (SNAP) are also involved in intracellular fusion events with SNAREs (Mayer *et al.* 1996). During protein transport, SNAREs form both *trans* (opposing membranes between donor and target vesicles, Figure 3a) and *cis* (same membrane, Figure 3b) complexes. The *trans* SNARE complexes are essential for vesicle interaction between vesicle-SNAREs (vSNARE) and target-SNAREs (tSNARE) in order to transport proteins. On the other hand, the c*is* SNARE complexes result from fusion of the opposing membranes (between donor and target vesicles) and participate in disassembly vesicles with SNAP and NSF (Weber *et al.* 1998; Allan *et al.* 2000; Cai *et al.* 2007).

SNAREs form α-helical coiled-coil complexes with highly conserved 15 hydrophobic layers arranged perpendicular to the axis of the helical bundle. Despite low sequence homology between yeast and human, SNAREs show similar secondary structures (Fasshauer *et al.* 1998). Generally, the SNARE complex is composed of one arginine SNARE (R-SNARE) which contains a central hydrophilic ‘0’-layer with arginine, and three glutamine SNAREs (Q-SNAREs), which contain a central hydrophilic ‘0’-layer with glutamine. As a consequence recent nomenclature divides the SNAREs into R-SNARE and Q-SNARE (Sutton *et al.* 1998). A conserved arginine or glutamine residue at the center of the SNARE domain is important for the

attachment of SNAP and NSF action. All known t-SNAREs are Q-SNAREs, and most v-SNAREs are R-SNAREs (Fasshauer *et al.* 1998). In the *T. cruzi*, SNARE proteins have highly conserved SNARE domains from yeast to human (-7 to 8 layers, figure 14) which are essential for forming SNARE complex and transmembrane domain in the C-terminus.

In the *P. tetraurelia*, Most *P. tetraurelia* synaptobrevins (PtSybs) are R-SNAREs and are located in the endoplasmic reticulum. PtSyb2 is found in the contractile vacuole complex such as the vacuole pore, contractile vacuole, collecting ampullae and canals. It is necessary for the structural integrity and function of the contractile vacuole complex (Schilde *et al.* 2006).

2.3.3 AP180

The monomeric assembly protein AP180 belongs to a group of clathrin assembly proteins. Clathrin-coated vesicles play important roles in transport between *trans*-Golgi network and endosomes, endocytosis at the plasma membrane and intracellular trafficking in neuronal cells. Clathrin-coated vesicles consist of clathrin triskelia, tetrameric adaptor proteins (AP), monomeric assembly protein (AP180), and numerous accessory proteins (Brodsky *et al.* 2001; Yao *et al.* 2005). AP180 was found in coated vesicles of bovine brain, and it is generally involved in membrane transport (McClure and Robinson 1996). The primary function of AP180 is to facilitate the assembly of clathrin-coated vesicles and to control the size of clathrin-coated vesicles (Meyerholz *et al.* 2005). In *Drosophila melanogaster* and *Caenorhabditis elegans,* the orthologues of AP180 are involved not only regulation of synaptic size but also sorting of synaptic proteins like synaptobrevin (Bao et al. 2005). In order to promote clathrin assembly, AP180 has an N-terminal homology domain (ANTH) that is highly conserved in all members of the AP180 family (Hao *et al.* 1999; Ford *et al.* 2001; Stavrou and O'Halloran 2006).

In the *T. cruzi*, the ANTH domain of the AP180 has 17~ 25% identities to homologous proteins in *Dictyostelium*, human and yeast.

In the *Dictyostelium discoideum,* AP180 was localized to punctae at the plasma membrane, the contractile vacuole, and the cytoplasm and associated with clathrin. AP180 *null* cells are osmosensitive and have especially large contractile vacuole complex. It has been suggested that AP180 has a role in the regulation of the contractile vacuole morphology and activity in *D. discoideum* (Stavrou and O'Halloran 2006).

2.3.4 Disgorgin/Drainin

DdDrainin is a peripheral membrane protein that was first functionally chracterized in *Dictyostelium*. DdDrainin and DdDisgorgin are required for proper contractile vacuole discharge and localizes to contractile vacuole bladders (Becker *et al.* 1999). Both sequences have ~44% identity and have TBC (Tre-2/Bub2/Cdc16) domain for GAP activity. DdDisgorgin's GAP activity is required for the cycling of Rab8A from the GTP-bound and the GDP-bound state. In addition, DdRab8A and DdDisgorgin contemporaneously localize to contractile vacuole bladders. DdDisgorgin and DdRab8A mediate the discharge of the contractile vacuole bladder by fusion between contractile vacuole and plasma membrane. On the contrary, DdDrainin does not have GAP activity because of lack of the conserved catalytic Arg and Gln residues in the TBC domain. But, DdDrainin is able to bind to DdRab11A-GTP as an effector of DdRab11A in vitro and acts in a signaling cascade in cooperative with a volume-sensing device in the contractile vacuole (Du *et al.* 2008).

In *T. cruzi* database, both TcDisgorgin and TcDrainin are annotated as a rab-like GTPase activating protein (RabGAP) and have same identity, but the result of sequence alignment with

DdDisgorgin and DdDrainin shows that TcDisgorgin/Drainin may be close to Disgorgin rather than Drainin because it has a TBC domain with conserved Arg and Gln residues and shows higher homology with DdDisgorgin.

2.3.5 Goal of this study

Taken together, the contractile vacuole complex has been known as an organelle involved in response to osmotic stress in *T. cuzi* but little is known about how membrane traffic contributes to the formation and function of this organelle. We hypothesized that proteins which play importenat roles in cell volume regulation are present in the contractile vacuole in *T. cruzi*. Here we report new contractile vacuole proteins in *T. cruzi* (TcV-ATPase subunits B and *a*, TcSNARE2.1 and 2.2, TcAP180, and TcDisgorgin/Drainin). By tagging the proteins with green fluorescence protein (GFP), we show that these proteins are localized to the contractile vacuole complex. We expect that these proteins are involved in regulatory volume decrease of contractile vacuole in *T. cruzi*.

MATERIALS AND METHODS

1. Parasites culture

T. cruzi epimastigotes CL strain were grown at 28 ^{o}C in liver infusion tryptose medium (LIT) supplemented with 5% heat inactivated newborn calf serum (NCS). Epimastigotes expressing GFP fusions were maintained in LIT medium supplemented with 10% heat inactivated NCS and 250 $\mu g \cdot ml^{-1}$ G418.

2. Cell extracts

T. cruzi epimastigotes (10^8) were harvested by centrifugation at 2,000 g for 5 min and washed twice with phosphate buffered saline (PBS, pH 7.4). Cell pellets were then resuspended in lysis buffer (50mM Tris-HCl, pH 7.4, containing mammalian protease inhibitor p8340 (1:250 dilution, Sigma Aldrich), 2 mM EDTA, and 2 mM PMSF). The cells were lysed by 3 cycles of freezing and thawing. Total extracts were centrifuged for 10 min at 13,000 g to separate a soluble fraction and a membrane-associated protein pellet. The pellet fraction was resuspended in PBS (pH 7.4) containing 1% SDS.

3. Cell treatments

For hypo-osmotic stress, cells were washed and resuspended in PBS (pH 7.4). The osmolarity of isotonic chloride buffer (137mM NaCl, 4mM KCl, 1.5mM KH_2PO_4, 8.5mM Na_2PO_4, 20mM Hepes, 11mM glucose, 1mM $CaCl_2$, 0.8mM $MgSO_4$) (Rohloff *et al.* 2003) was

adjusted to 300 mOsm as verified by an Advanced Instruments 3D3 Osmometer (Norwood). A 50% hypo-osmotic stress was induced by a 1:1 dilution of cell suspensions in the isotonic chloride buffer with deionized water.

3. *Cloning of contractile vacuole genes*

To examine the distribution of contractile vacuole proteins in *T. cruzi*, the genes (Table 1) encoding TcV-ATPase B, TcV-ATPase *a,* TcSNARE2.1, TcSNARE2.2, TcAP180, TcRab11, TcDisgorgin/Drainin were amplified with gene specific primers (Table 2). The PCR was performed with 30 cycles of 95 ^{o}C for 30 sec for denaturation, 55 ^{o}C for 30 sec for annealing, and 72 ^{o}C for 1~3 min for extension, using 1.5 units of *pfu* Ultra High-Fidelity DNA polymerase (Stratagene) with 400 ng of *T. cruzi* genomic DNA, 25 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.2 mM each dNTP in a total volume of 20 µl. PCR products were cloned into the pCR-Blunt II-TOPO (Invitrogen) and subcloned sequences were confirmed by sequencing (Yale DNA Analysis Facilities).

4. GFP fusion constructs

We generated constructs for over-expression. The cloned genes were subcloned into *T. cruzi* an expression vector to fuse the N-terminal or C-terminal region of the genes to GFP. A green fluorescence protein (GFP) was subcloned at N-terminal GFP tagging and at C-terminal GFP tagging. The *T. cruzi* contractile vacuole proteins encoding genes subcloned into pCR-BluntII-TOPO were moved into pTEX-GFP vectors at each restriction enzyme sites, respectively.

5. Generation of cells over-expressing contractile vacuole-GFP proteins

Transfections were performed in a 2-mm gap cuvette with a Bio-Rad Gene Pulser II set at 0.3 kV and 500 μF. 10^8 parasites were harvested, washed with PBS buffer (pH 7.2), and resuspended in 0.4 ml electroporation buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM Hepes, 2 mM EDTA, 5 mM $MgCl_2$, pH 7.6) with 80 μg of plasmid DNA. The parasites were recovered in 5 ml of LIT medium supplemented with 10% fetal calf serum at 28 ^oC. After 24 hr, G418 was added to a final concentration 250 $\mu g \cdot ml^{-1}$, and selected for 40 days.

6. Immunofluorescence microscopy

To determine intracellular location of contractile vacuole proteins, we analyzed the GFP signal of cells expressing TcCV-GFP under both iso-osmotic condition (300 mOsm) and hypo-osmotic stress (150 mOsm) using fluorescence microscopy. For immunofluorescence, cells were fixed with 4% paraformaldehyde, adhered to poly-L-lysine coverslips, permeabilized for 5 min with Dulbecco's phosphate buffered saline (PBS), 0.3% Triton X-100, blocked for 1 hr (PBS pH 7.4, 3% bovine serum albumin, 1% fish gelatin, 5% goat serum, 50 mM NH_4Cl), incubated with primary antibody for 1 hr, and incubated with secondary antibody for 1 hr. For visualization of calmodulin, goat polyclonal anti-calmodulin antibody (1:500 dilution, Santa Cruz Biotechnology) was used, followed by rabbit anti-goat Alexa 546 conjugate (1:2000 dilution, Molecular Probes). Specimens were observed with a Delta vision restoration system.

7. Western blot analysis

Cell lysates were obtained from 10^8 epimastigotes, and proteins were separated in 10% SDS-PAGE and blotted onto nitrocellulose membranes. Filters were processed at room

temperature. The following steps were performed in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05 % Tween 20). Membranes were blocked overnight in 5% nonfat dry milk and washed three times, incubated with polyclonal anti-GFP antibody (1:5,000 dilution, Molecular Probes) for 1 hr and washed three times, incubated with goat anti-rabbit horseradish peroxidase (1:15,000 dilution, Molecular Probes) for 1 hr and washed three times. The ECL western blotting kit (Pierce) and X-ray film (Agfa) were employed to detect the immunecomplexes.

8. Expression and purification of recombinant proteins in E. coli

8.1 TcV-ATPase B recombinant protein for generation of a specific antibody

The entire TcV-ATPase B gene was sub-cloned into pCR2.1-TOPO cloning vector (Invitrogen). The gene was excised with BamHI (New England Biolabs) and XhoI (New England Biolabs) and cloned into pET28a (Novagen) to produce His tagged protein (pET28a-TcV-ATPase B). The construct pET28a-TcV-ATPase B was transformed into BL21 Codon Plus host, and the recombinant protein was induced with 500 μM isopropyl-1-thio-β-D-galactopyranoside at 18 ^{o}C overnight. The TcV-ATPase B-His tagged protein was insoluble. Therefore, this recombinant protein was purified under denaturing condition. The induced 1 L cultured bacterial cells were harvested by centrifugation, and resuspended in lysis buffer (6 M guanidinium chloride, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 8.0). The protein extract was prepared by sonication at 30% amplitude for 3 min. This fraction was purified using Ni-NTA His bind resin (Novagen) and eluted with 10 ml of elution buffer (8M urea, 0.1 M $Na_2H_2PO_4$, 0.01 M Tris-HCl pH 4.5). The purified recombinant protein band was sent to Cocalico Biologicals for generation of a specific antibody in a rabbit. The rabbit was ex-sanguinated after four boosts.

8.2 TcSNARE2.1 recombinant protein for generation of a specific antibody

The C-terminus transmembrane domain (541 ~ 630 nucleotides) deleted TcSNARE2.1 gene was subcloned into pCR2.1-TOPO cloning vector (Invitrogen). The gene was excised with BamHI (New England Biolabs) and XhoI (New England Biolabs) and cloned into pET28a (Novagen) to produce His tagged protein (pET28a-TcSNARE2.1). The construct pET28a-TcSNARE2.1 was transformed into BL21 Codon Plus host, and recombinant protein was induced with 500 μM isopropyl-1-thio-β-D-galactopyranoside at 18 ^{o}C overnight. The TcSNARE2.1-His tagged protein was soluble. The induced 1 L cultured bacterial cells were harvested by centrifugation, resuspended in lysis buffer (5mM imidazole, 0.5M NaCl, 20mM Tris-HCl, pH7.9). The protein extract was prepared by sonication at 30% amplitude for 3 min. This fraction was purified using Ni-NTA His bind resin (Novagen) and eluted with 10 ml elution buffer (1M imidazole, 0.5M NaCl, 20mM Tris-HCl, pH 7.9). The purified recombinant protein was sent to Cocalico Biologicals for generation of a specific antibody in rats. The rats were ex-sanguinated after four boosts.

RESULTS

1. Contractile vacuole proteins in *T. cruzi*

Although recent studies showed that the contractile vacuole is involved in osmoregulation in *T. cruzi*, the volume regulatory role of the contractile vacuole in *T. cruzi* during fluctuation in osmolarity is poorly understood. In a previous proteomic study in the laboratory, several proteins with homology to protozoan contractile vacuole proteins were identified in a subcellular fractions enriched in contractile vacuoles of *T. cruzi* (Table 1, Ulrich et al. unpublished)

V-H^+-ATPase, LvsA (Beige/LYST family) (Heuser *et al.* 1993; Gerald et al. 2002; Wu et al. 2004), Rab11, and drainin/disgorgin are known to regulate the contractile vacuole system and the pathways that control contractile vacuole discharge in *D. discoideum* (Becker et al. 1999; Du et al. 2008). Clathrin and AP180 also localize to the contractile vacuole and are required for biogenesis of the contractile vacuole complex in *D. discoideum* (Stavrou and O'Halloran 2006). Golvesin is also found in *Dictyostelium* contractile vacuole. SNAREs are intracellular trafficking proteins that localize to the contractile vacuole and are involved in the function of the contractile vacuole in *P. tetraurelia* (Schilde et al. 2006). In this study, we examined the localization of TcV-ATPase subunit B and TcV-ATPase subunit *a*, TcSNARE2.1, TcSNARE2.2, TcAP180, and TcDisgorgin/Drainin.

2. TcV-H^+-ATPase subunit B associates with the contractile vacuole bladder

The 1491 nucleotides sequence of *T. cruzi* V-ATPase subunit B (TcV-ATPase B) encoding gene (Tc00.1047053506025.50) contained one open reading frame. A BLAST search of the protein databases (TriTrypDB, http://tritrypdb.org/tritrypdb) revealed that TcV-ATPase B has greater than 70% identity to homologous proteins in *Dictyostelium*, *S. cerevisiae*, and human (Figure 4).

To examine the distribution of TcV-ATPase B, we tagged TcV-ATPase B at the N-terminus with GFP in the BamHI and HindII sites of the pTEX expression vector (pTEX-TcV-ATPase B-GFP) (Figure 5).

TcV-ATPase B-GFP was detected in a vacuole that seemed like the contractile vacuole in iso-osmotic buffer (300mOsm) (Figure 6A). To enlarge the bladder of the contractile vacuole for easy detection, live cells were placed in hypo-osmotic buffer (150 mOsm) for 5 min. TcV-ATPase localizes to the membrane of the bladder of the contractile vacuole by fluorescence microscopy (Figure 6B). We also carried out immunofluorescence assays to confirm the distribution of TcV-ATPase B using human calmodulin antibody (Molecular Probes) as a marker for the spongiome. TcV-ATPase B was observed in the contractile vacuole, but did not co-localize with calmodulin (Figure 6C). As a negative control we incubated cells without primary antibodies, with no reaction (data not shown).

Proper tagging of TcVATPase B was confirmed by western blot with GFP antibody (Molecular Probes) in a membrane fraction prepared as described in Materials and Methods. We detected an 80 kDa (55 kDa V-ATPase B + 25 kDa GFP) band (Figure 7). The TcV-ATPase B protein is distributed in soluble and membrane fractions.

Antibodies against the entire TcV-ATPase B were raised in a rabbit. Recombinant TcV-ATPase B antibodies were fused to a 6X-His tag and affinity purified as described in Materials and Methods (Figure 8). These antibodies recognized the recombinant protein (~60 kDa), but could not detect any protein in epimastigotes over-expressing TcV-ATPase B (Figure 9). In immunofluorescence assays, the pattern of α-TcV-ATPase B staining was punctate throughout the cytoplasm (data not shown).

3. TcV-H^+-ATPase subunit *a* (TcV-ATPase *a*) associates with the contractile vacuole bladder

BLAST search of the TcV-ATPase subunit *a* gene (Tc00.1047053509601.70, 2322 nucleotides) showed that TcV-ATPase subunit *a* gene has ~40% identity to orthologues in *Dictyostelium*, human, and yeast (Figure 10).

To localize of TcV-ATPase *a*, we generated constructs of TcV-ATPase *a* tagged at the N-terminus with GFP in the BamHI and HindIII sites of the pTEX expression vector (pTEX-TcV-ATPase *a*-GFP) (Figure 11).

TcV-ATPase *a* was detected in the contractile vacuole of live *T. cruzi* epimastigote incubated under iso-osmotic buffer (300 mOsm) (Figure 12A). To enlarge the bladder of the contractile vacuole for easy detection of contractile vacuole, live cells were incubated for 5 min in a hypo-osmotic buffer (150 mOsm). This treatment swells the cells and to recover the contractile vacuole becomes encouraged because it collects H_2O. TcV-ATPase *a*-GFP localize in the bladder membrane of the contractile vacuole by fluorescence microscopy (Figure 12B). We also carried out immunofluorescence assay to confirm the distribution of TcV-ATPase subunit *a* using human calmodulin antibody (Molecular Probes) as one of the contractile vacuole spongiome markers . TcV-ATPase *a* was observed in the contractile vacuole, but did not co-

localize with calmodulin (Figure 12C). As a negative control we incubated cells without primary antibodies (data not shown).

Labeling of TcV-ATPase *a*-GFP in *T. cruzi* was confirmed by western blot analysis with GFP antibody (Molecular Probes) in a membrane fraction prepared as described in Materials and Methods. We could detect a 110 kDa of TcV-ATPase *a*-GFP protein (85 kDa V-ATPase *a* + 25 kDa GFP) band (Figure 13).

4. TcSNARE2.1 and TcSNARE2.2 associate with the spongiome of the contractile vacuole

BLAST searches of TcSNARE2.1 (Tc00.1047053507625.183, 630 nuclotides) and TcSNARE2.2 (Tc00.1047053506715.50, 648 nucleotide) revealed that the amino acid sequence encoded by TcSNARE2 genes have greatest homology to PtSyb2 (*Paramecium tetraurelia*) and representative mammalian R-SNAREs. Both TcSNARE2.1 and TcSNARE2.2 have a highly conserved arginine residue in the central layer and SNARE domains. The layers consist of leucine, isoleucine and valine residues and follow the packing characteristics of parallel, tetrameric leucine-zipper proteins (Sutton *et al.* 1998). A central hydrophilic '0'-layer contains arginine residue at the complex core. R-SNAREs or v-SNAREs have conserved transmembrane domain for membrane targeting (Figure 14).

To examine the distribution of TcSNARE2s, we generated constructs with TcSNARE2s tagged at the N-terminus with GFP in the BamHI and HindII sites of pTEX expression vector (pTEX-TcSNARE2.1-GFP and pTEX-TcSNARE2.2-GFP) (Figure 15).

TcSNARE2.1-GFP and TcSNARE2.2-GFP localize to near the contractile vacuole under iso-osmotic buffer (300mOsm) (Figure 16A and Figure 17A). To enlarge the bladder of contractile vacuole for easy detection of contractile vacuole, the live cells were placed in hypo-

osmotic buffer (150mOsm) without fixation for 5 min and observed. TcSNARE2.1-GFP and TcSNARE2.2-GFP were localized in the side of the contractile vacuole bladder by fluorescence microscopy (Figure 16B and Figure 17B). We performed immuno-fluorescence assay to confirm the distributions of TcSNARE2.1-GFP and TcSNARE2.2-GFP using α-human calmodulin antibody, a marker for the contractile vacuole spongiome. As a result, both TcSNARE2.1-GFP and TcSNARE2.2-GFP co-localized with calmodulin (Figure 16C and Figure 17C).

Immunolocalizations of TcSNARE2.1-GFP and TcSNARE2.2-GFP in *T. cruzi* were confirmed by western blot with GFP antibody (Molecular Probes) in membrane fractions prepared as described, which detected a band of approximately 50 kDa (25 kDa of TcSNARE2.1-GFP and TcSNARE2.2-GFP and 25 kDa of GFP) (Figure 18). Western blot analysis revealed that most of the TcSNARE2.1-GFP and TcSNARE2.2-GFP proteins are membrane associated.

We attempted to produce an antibody against TcSNARE2.1 in two rats using affinity purified recombinant TcSNARE2.1 fused to a 6X-His tag as an immunogen (Figure 19). The antibodies were able to recognize a TcSNARE2.1 protein in epimastigotes over-expressing TcSNARE2.1-GFP (~50 kDa) and TcSNARE2.1 recombinant protein (~50 kDa) but were not able to detect the TcSNARE2.1 in wild type epimastigote extracts (Figure 20). In addition, immunofluorescence microscopy with these antibodies was unsuccessful (data not shown).

5. TcAP180 associates with the contractile vacuole bladder

The 1503 nucleotides sequence of TcAP180-encoding gene (Tc00.1047053509875.190) was identified and BLAST searches revealed that members of the AP180 family have at their N-terminus a signature domain called ANTH (AP180 N-terminal homology) domain. Analysis of

TcAP180 sequence showed a conserved lysine-rich motif *KVTxxxxxPKxKH* at the N-terminus, which agreed with the ANTH domain consensus sequence *(K/G)A(T/I)xxxxxx(P/L/V)KxK(H/Y)* (Kay *et al.* 1999; Ford *et al.* 2001). The first 300 amino acids at the N-terminus shared significant homology with the ANTH domain of the AP180 orthologues from *Dictyostelium* (ClmA; GenBank ID, DDB0235311; 25% identity), human (SNAP91; GenBank ID 9892; 26% identity), and yeast (YAP1801; GenBank ID 856566; 17% identity) (Figure 21).

To examine the distribution of TcAP180, we generated the construct TcAP180 tagged at the C-terminus with GFP in BamHI/XhoI sites of pTEX (pTEX-GFP-AP180) (Figure 22).

The fluorescence of GFP-TcAP180 appeared to localize to a vacuole that looks like the contractile vacuole in iso-osmotic buffer (300 mOsm) (Figure 23A). To enlarge the bladder of contractile vacuole for easy detection of contractile vacuole, the live cells were incubated for 5 min in hypo-osmotic buffer (150 mOsm). We detected that TcAP180 localized to the bladder membrane of the contractile vacuole by fluorescence microscopy (Figure 23B). We also carried out immunofluorescence assays to confirm the distribution of TcAP180 using human calmodulin antibody (Molecular Probes) as one of the contractile vacuole spongiome markers. The TcAP180 was observed in the contractile vacuole, but did not co-localize with calmodulin (Figure 23C).

Proper tagging of TcAP180 was confirmed by western blot analysis with GFP antibody (Molecular Probes) in membrane fractions prepared as described. We detected an 80 kDa (55 kDa TcAP180 + 25 kDa GFP) band. The AP180 protein is distributed in both soluble and membrane fractions by separation of protein extract (Figure 24).

6. TcDisgorgin/Drainin is a Peripheral Membrane Protein Associated with the Flagellar Pocket

TcDisgorgin/Drainin encoding gene (Tc00.1047053508723.80, 1236 nuleotides) was identified and BLAST searches of the protein databases revealed that the amino acid sequence encoded by TcDisgorgin/Drainin gene shows 17% identity to *Dictyostelium* Drainin and 27% identity to *Dictyostelium* Disgorgin (Figure 25). In *Dictyostelium*, both Drainin and Disgorgin contain a TBC (Tre-2/Bub2/Cdc16) domain for GAP activity. DdDrainin lacks the conserved catalytic Arg and Gln required for Rab GAP activity in the TBC (Bos et al, 2007) whereas DdDigorgin has those residues for Rab8A GAP activity. The result of multiple sequence alignment showed that TcDisgorgin/Drainin has both Arg and Gln residues in the TBC domain. Therefore, we speculated that the identity of TcDisgorgin/Drainin is close to Disgorgin rather than Drainin and may have Rab GAP activity.

To examine the distribution of TcDisgorgin/Drainin, we generated the constructs TcDisgorgin/Drainin tagged at the N-terminus with GFP in XbaI and EcoRV sites of pTREX expression vector (pTREX-TcDisgorgin/Drainin-GFP). (Figure 26).

We observed the majority of TcDisgorgin/Drainin in the cytosol. A small proportion concentrates anterior to the kinetoplast in a region near the flagellar pocket (Figure 27A). *T. cruzi* epimastigotes over-expressing tagged disgorgin were markedly more sensitive to hypo-osmotic stress (150 mOsm) than wild-type epimastigotes or epimastigotes expressing GFP alone (Figure 27A). The over-expression cell lines failed to develop functional contractile vacuoles and volume recovery required more time than with wild-type cells. TcDisgorgin/Drainin partially co-localized with concanavalin A (Sigma Aldrich, ConA), a marker for the flagellar pocket (Figure 27C), but did not co-localize with calmodulin, a marker for the spongiome (Figure 27B). Western

blots probed with GFP antibodies revealed that TcDisgorgin/Drainin was present in both soluble and membrane-associated fractions (Figure 28).

DISCUSSION

The contractile vacuole is a specialized organelle needed in protists to live both under hypo-osmotic and hyper-osmotic conditions. It consists of two components: a central contractile vacuole bladder and a vesicular spongiome surrounding the bladder. In a previous study, ~220 proteins from fractions enriched in contractile vacuoles from *T. cruzi* epimastigote were identified by proteomic analysis. Of these contractile vacuole proteome, twelve *T. cruzi* contractile vacuole proteins with great homology to contractile vacuole proteins in *Dictyostelium* and *Paramecium* were selected (Table 1, Ulrich et al. unpublished).

In this study, we report six proteins localized in the contractile vacuole complex of *T. cruzi*, TcV-ATPase subunit B, TcV-ATPase subunit *a*, TcSNARE2.1, TcSNARE2.2, TcAP180, and TcDrainin. Our results reveal that most of these proteins are associated with the contractile vacuole, but TcDrainin is found in the cytosol and the flagellar pocket in the vicinity of contractile vacuole.

Localization of the TcV-H^+-ATPase B and *a* subunits

V-ATPases on the contractile vacuole complex, a classical marker for contractile vacuole in protozoa, are important for formation of an osmotic gradient and allow the transfer of water from inside the cell to the contractile vacuole bladder. *In Dictyostelium*, V-ATPase is found in tubular membrane around the contractile vacuole complexes. *In Paramecium*, it is localized in the decorated, round and smooth spongiome of the contractile vacuole complex.

In addition, V-ATPase on the contractile vacuole complex plays a general and important role in contractile vacuole function and integrity of contractile vacuole structure. V-ATPase subunit B is an essential component of V_1 subcomplex of the V-ATPase, which is responsible for hydrolysis of ATP. TcV-ATPase B has more than 70% identity to homologous proteins in *Dictyostelium*, *S. cerevisiae*, and human. By GFP tagging, we detected that V-ATPase B is localized in the bladder of the contractile vacuole. Under hypo-osmotic conditions, TcV-ATPase B showed clear localization to the top margin of the bladder membrane (Figure 6B). We performed immunofluorescence to confirm the distribution of TcV-ATPase B by using human α-calmodulin antibody as a marker for the contractile vacuole spomgiome. Calmodulin which is calcium binding protein was found to be associated with contractile vacuole membrane and its periphery in *P. tetraurelia* (Momayezi *et al.* 1986) and *D. discoideum* (Zhu and Clarke 1992). It was consistent with a role for contractile vacuole in calcium homeostasis elucidated in *Dictyostelium* (Nolta *et al.* 1991). Therefore, calmodulin has been used as a contractile vacuole marker.

TcV-ATPase B was observed in the contractile vacuole, but did not co-localize with calmodulin (Figure 6C). On the other hand, V-ATPase subunit *a* that is a component of V_0 subcomplex plays a crucial role in coupling ATP hydrolysis to proton translocation. By GFP tracking under hypo-osmotic condition and using antibody against human calmodulin, we detected that TcV-ATPase *a*-GFP was found in the membrane of the bladder of the contractile vacuole (Figure 12). Consequently, TcV- ATPase B and *a* subunits localize in the bladder membrane. We postulate that the gradient generated by V-ATPase may facilitate the generation of an osmotic gradient to remove water from the cell in *T. cruzi* as well as *Dictyostelium* (Giglione and Gross 1995).

Localization of the TcSNARE2.1 and TcSNARE2.2

SNAREs mediate fusion of intracellular vesicles with other vesicles or cell membranes for membrane trafficking. In *P. tetraurelia*, Ptsyb2, which is an R-SNARE, was found in the contractile vacuole complex such as the vacuole pore, collecting ampullae and canals. It is necessary for the structural integrity and function of the contractile vacuole complex. Two TcSNARE2 proteins identified in the proteomics data, which we called TcSNARE2.1 and TcSNARE2.2 co-localized with calmodulin in the spongiome (Figure 16 and Figure 17). These SNAREs could be involved in fusion of the spongiome with the membranes of the bladder or with other organelles in the cell. Acidocalcisomes, acidic organelles containing calcium and large amounts of phosphate in the form of polyphosphate, associate with the contractile vacuole of *T. cruzi* during hypo-osmotic stress. Fusion of the contractile vacuole with acidocalcisomes may be mediated by interactions among SNAREs.

Localization of the TcAP180

The adaptor protein AP180 facilitates the assembly of clathrin-coated vesicles and controls the size of clathrin-coated vesicles. In *Dictyostelium,* AP180 was localized to punctae at the plasma membrane, the contractile vacuole, and the cytoplasm and was associated with clathrin. AP180, an adaptor for clathrin in the contractile vacuole, was involved in vacuolar fusion in the contractile vacuole and functions with clathrin in the regulation of contractile vacuole size. It has been suggested that AP180 has important role for regulation of contractile vacuolemorphology and activity. *T. cruzi* AP180 has an N-terminal homology domain (ANTH) that is highly conserved in all members of AP180 family to promote clathrin assembly. Using fluorescence microscopy, we detected that the TcAP180 was present in the contractile vacuole

bladder, but did not co-localize with calmodulin (Figure 23). Therefore, we postulate that TcAP180 localized to the contractile vacuole bladder and may be involved in vacuolar fusion of the contractile vacuole.

Localization of the TcDisgorgin/Drainin

TcDisgorgin is annotated as a rab-like GTPase activating protein (RabGAP). It has a conserved TBC (Tre-2/Bub2/Cdc16) domain with conserved arginine (R198) and glutamine (Q235) residues important for RabGAP activity (Bos *et al.* 2007). Disgorgin may play a role in targeting the fusion of the contractile vacuole bladder to the flagellar pocket during discharge. Epimastigotes over-expressing tagged TcDisgorgin/Drainin were sensitive to hypo-osmotic shock. Commonly, TcDisgorgin/Drainin-GFP accumulated on large vesicles at the posterior end of epimastigotes. Interestingly, knock-outs of disgorgin in *D. discoideum* develop large vacuoles as well (Du *et al.* 2008). Our observation that only a small proportion of disgorgin associates with the flagellar pocket is consistent with a role as a peripheral membrane protein and observations in *Dictyostelium* that only 5% of disgorgin associates with the contractile vacuole (Du *et al.* 2008).

CONCLUSION

Contractile vacuole is an essential component of the volume machinery in *T. cruzi*. We have demonstrated the presence of six new contractile vacuole proteins in *T. cruzi* using proteomic analysis and overexpression of the contractile vacuole proteins with GFP fusion *in T. cruzi*.

Table 1. Contractile vacuole proteins in *T. cruzi.*

Gene	Gene ID	Protists with homologous CV proteins	References
TcV-H$^+$-ATPase subunit B*	**Tc00.1047053506025.50**	***D. discoideum***	**Heuser et al 1993**
TcV-H$^+$-ATPase subunit *a**	**Tc00.1047053509601.70**	***D. discoideum***	**Liu and Clarke 1996**
TcSNARE2.1*	**Tc00.1047053507625.183**	***P. tetraurelia***	**Schilde et al 2006**
TcSNARE2.2*	**Tc00.1047053506715.50**	***P. tetraurelia***	**Schilde et al 2006**
TcAP180*	**Tc00.1047053509875.190**	***D. discoideum***	**Stavrou and O'Halloran 2006**
TcDisgorgin/Drainin*	**Tc00.1047053508723.80**	***D. discoideum***	**Du et al 2008**
TcRab11	Tc00.1047053511407.60	*D. discoideum*	Du et al 2008
TcGolvesin	Tc00.1047053509805.40	*D. discoideum*	Schneider et al 2000
	Tc00.1047053503455.30	*D. discoideum*	Schneider et al 2000
TcMyosin V heavy chain	Tc00.1047053511527.70	*A. castellani*	Baines et al 1992
TcMyosin IB heavy chain	Tc00.1047053507739.110	*A. castellani*	Baines et al 1992
TcClathrin	Tc00.1047053506167.50	*D. discoideum*	Stavrou and O'Halloran 2006
TcLvsA	Tc00.1047053508239.30	*D. discoideum*	Gerald et al 2002

* *T. cruzi* contractile vacuole proteins identified by proteomic analysis were examined in this study.

Table 2. PCR primers

Gene	Primers	Primer sequences
TcV-H^+-ATPase subunit B	Forward	5'-GGATCCATGGGCATACATGAGGCAGAGGAG-3'
	Reverse	5'-AAGCTTCTTCCGCTCGGGTTGGCGGTCGTAG-3'
TcV-H^+-ATPase subunit *a*	Forward	5'-GGATCCATGCCACGTGAAGCCGCCAGCGG-3'
	Reverse	5'-AAGCTTGTTAATTTTGCTAAGAACCTCTGC-3'
TcSNARE2.1	Forward	5'-GGATCCATGCTTTTTTTTACTCTTATCGTC-3'
	Reverse	5'-AAGCTTTGCCAAAGCGGCATAGTAAAATATG-3'
TcSNARE2.2	Forward	5'-GGATCCATGGTGACGATTCGTTACGCCCTTG-3'
	Reverse	5'-AAGCTTATTTCTTTTGCAGCGATTAAAGTTG-3'
TcAP180	Forward	5'-GGATCCATGAATGTGAAAGATTCTAATGAACTG-3'
	Reverse	5'-CTCGAGCTAAATGTTATTGGCATGCC-3'
TcDisgorgin/Drainin	Forward	5'-TCTAGAATGCAGGAGGGTAGCGTCTTTGGG-3'
	Reverse	5'-GATATCCGCCGCTCTCTGTTCGCGCCAATA-3'

Full coding sequences for all genes were amplified by polymerase PCR using above specific oligonucleotides.

Figure 1. Model proposed for regulatory volume decrease in *T. cruzi*. A. Contractile vacuole shows limited activity and normal size when cells are in iso-osmotic conditions**.** B. When cells are placed in hypo-osmotic conditions, contractile vacuole is rapidly activated. Cell swelling leads to activation of adenylyl cyclase and protein kinase. Acidocalcisomes fuse to the contractile vacuole with translocation of an aquaporin as a water channel to facilite water transport. Acidocalcisomes release amino acids, Ca^{2+}, and inorganic phosphate into the contractile vacuole. Water goes into the contractile vacuole through the aquaporin. Water is ejected into the flagellar pocket. This cartoon is adapted from Rohloff and Docampo (2008).

Figure 1.

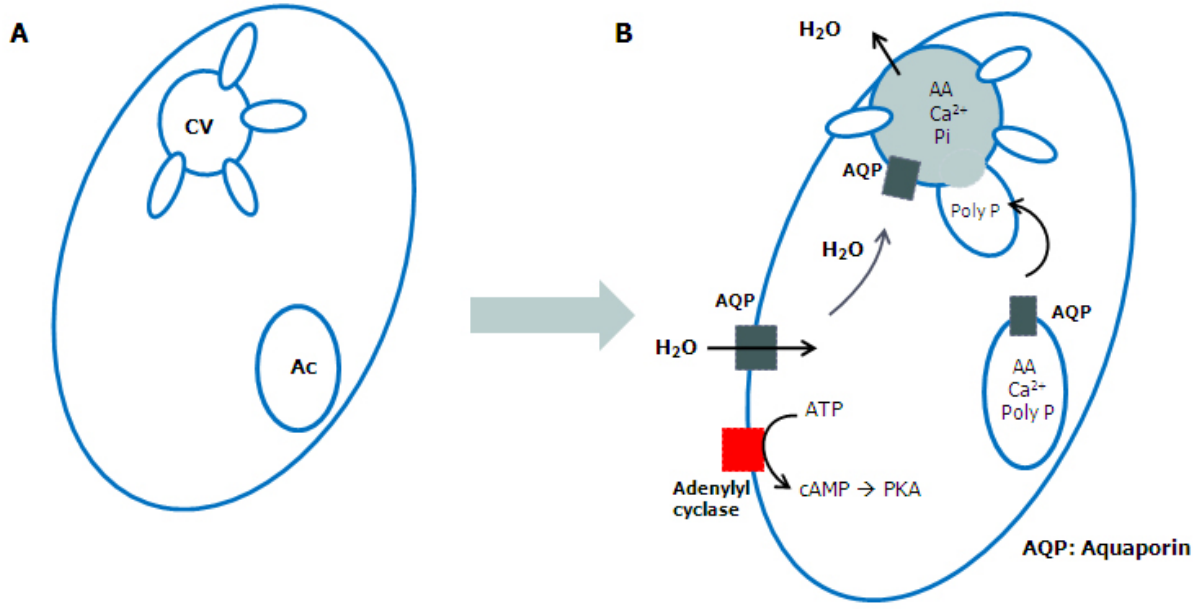

A
CV
Ac
B
H2O
AA
Ca2+
Pi
AQP
Poly P
H2O
AQP
H2O
AQP
AA
Ca2+
Poly P
ATP
Adenylyl
cyclase
cAMP → PKA
AQP: Aquaporin

Figure 2. Model for the V-H^+-ATPase expressed in a eukaryotic cell membrane. The peripheral V_1 complex consists of eight different subunits identified with capital letters A-H. Subunit G exists as the dimer G_2. The integral membrane V_0 complex is composed of at least four different subunits identified with small letters *a, c, d, e*. This cartoon is adapted from (Beyenbach and Wieczorek 2006).

Figure 2.

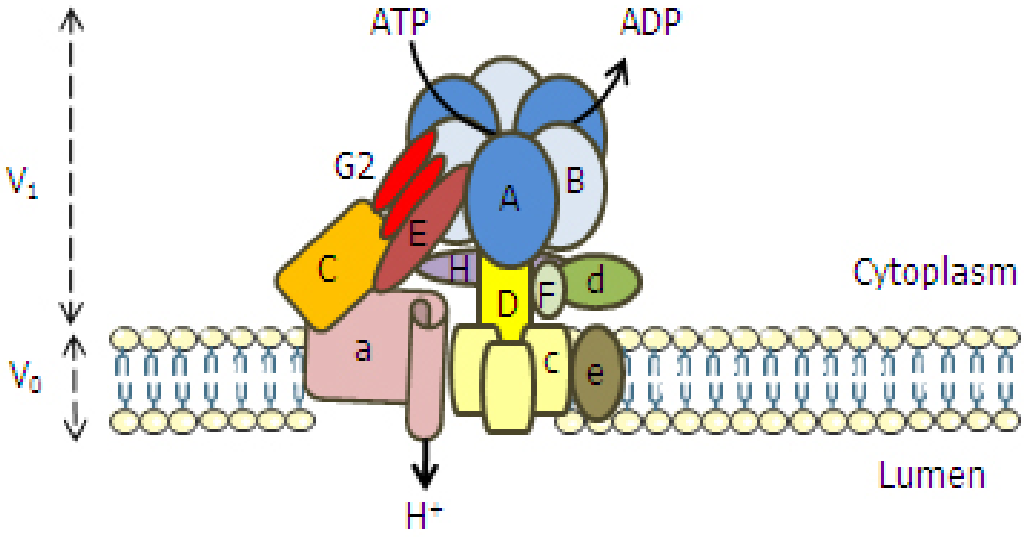


Figure 3. Overview of assembly and dissembly of SNARE complexes. A. vesicle-SNARE and target-SNARE on the acceptor membrane assemble into a four-helix bundle (trans-SNARE complex between vesicle and target membranes), which drives membrane fusion and the delivery of cargo (cartoon adapted from Cai, H et. al., 2007). B. SNAP association with the cis-SNARE complex (SNARE complex in the target membrane) enables NSF binding to the SNAP-SNARE complex. Stimulation of NSF ATPase activity leads to dissembly of the complex and v-SNARE move back (cartoon adapted from Morgan, A et.al., 2004).

Figure 3.

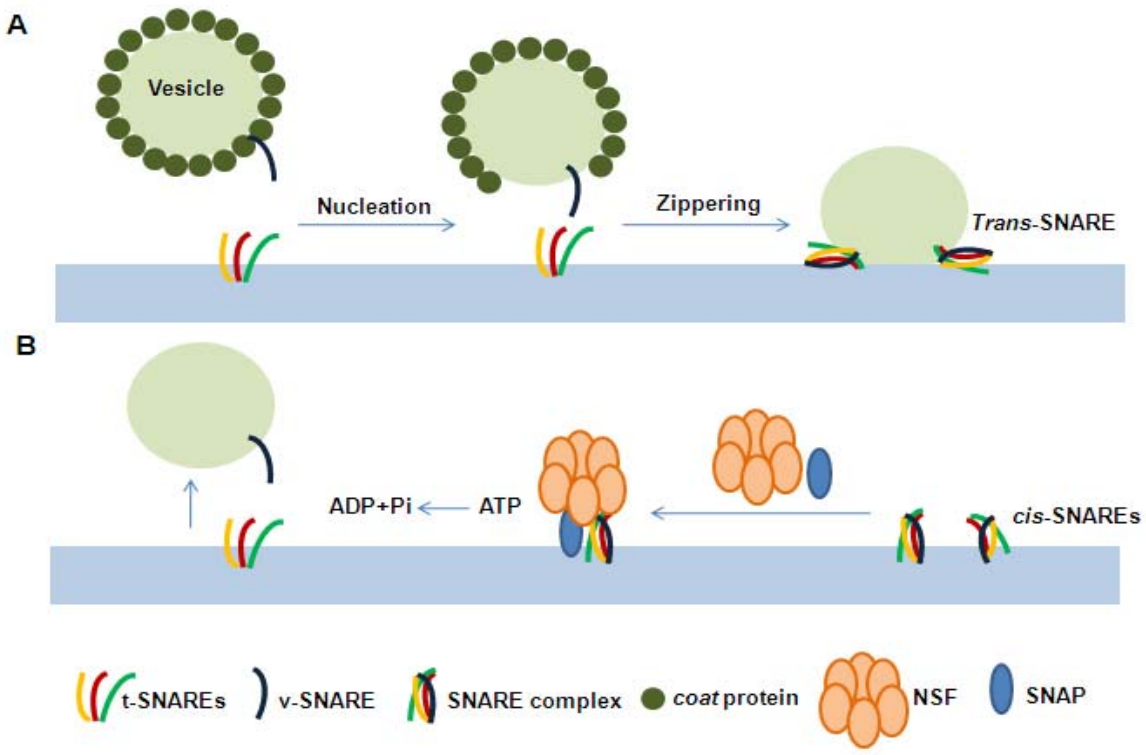
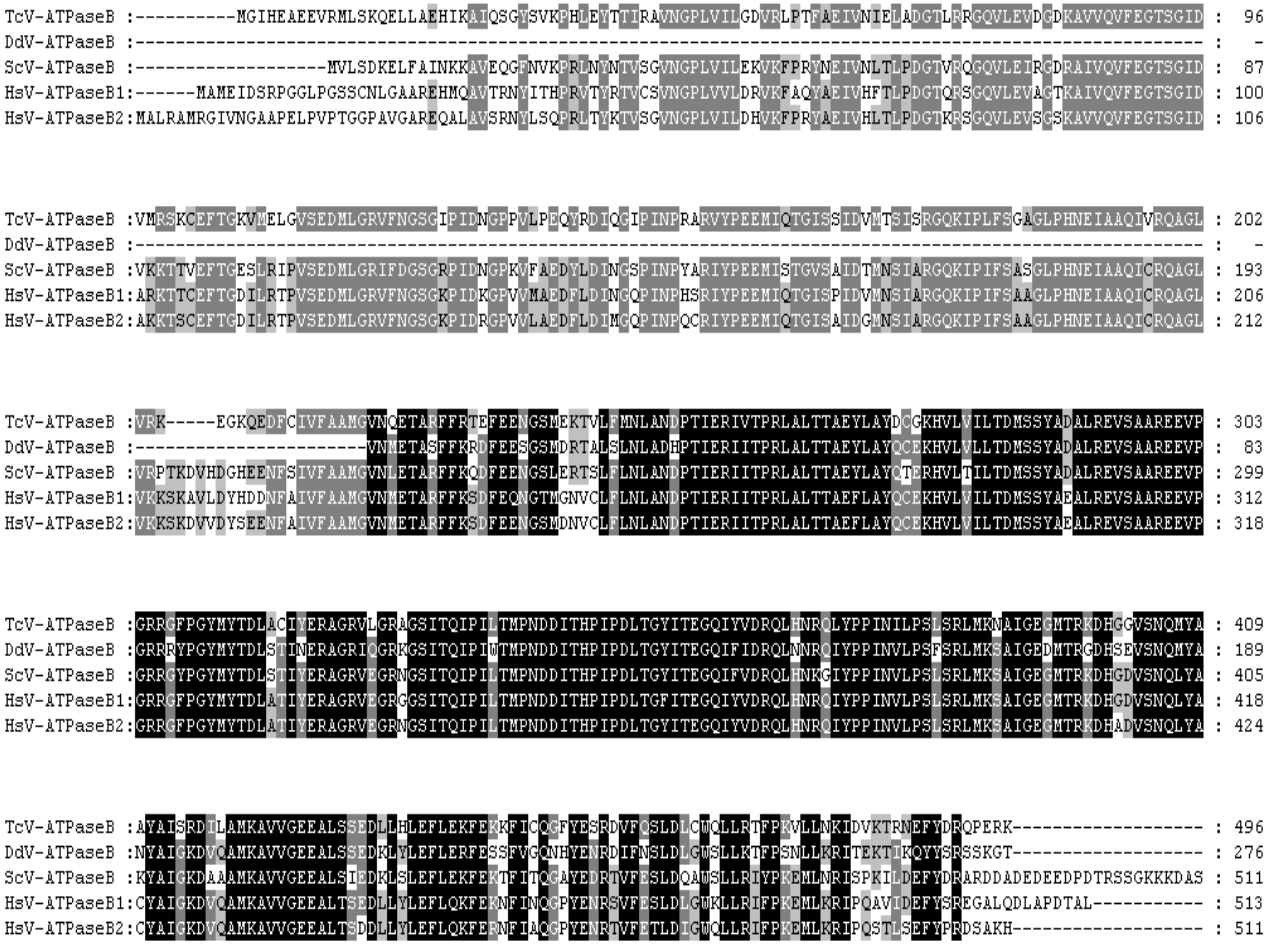

A
Vesicle
Nucleation
Zippering
Trans-SNARE
B
ADP+Pi
ATP
cis-SNAREs
t-SNAREs
v-SNARE
SNARE complex
coat protein
NSF
SNAP

Figure 4. Sequence alignments of TcV-ATPase subunit B. Alignment was performed using ClustalW. Identical amino acids are shown in black boxes. Conserved changes are shown in light and dark gray boxes. Numbers correspond to amino acid positions in each polypeptide. The amino acid sequence for the *T.cruzi* V-ATPase subunit B, the *Dictyostelium* V-ATPase subunit B (VatB; GeneBank ID, DDB0185207), *S. cerevisiae* V-ATPase subunit B (Vma2; GenBank ID,852424), and Human V-ATPase subunit B1 (ATP6V1B1; GenBank ID, 525) and B2 (ATP6V1B2; GenBank ID, 526) are shown.

Figure 4.


TcV-ATPaseB :----------MGIHEAEEVRMLSKQELLAEHIKAIQSGVSVKPHLEYTTIRAVNGPLVILGDVRLPTFAEIVNIELADGTLRRGQVLEVDGDKAVVQVFEGTSGID : 96
DdV-ATPaseB :---------- : -
ScV-ATPaseB :-------------------MVLSDKELFAINKKAVEQGTNVKPRLNYNTVSGVNGPLVILEKVKFPRYNEIVNLTLPDGTVRQGQVLEIRGDRAIVQVFEGTSGID : 87
HsV-ATPaseB1:------MAMEIDSRPGGLPGSSCNLGAAREHMQAVTRNVITHPRVTYRTVCSVNGPLVVLDRVKFAQYAEIVHFTLPDGTQRSGQVLEVAGTKAIVQVFEGTSGID : 100
HsV-ATPaseB2:MALRAMRGIVNGAAPELPVPTGGPAVGAREQALAVSRNVLSQPRLTYKTVSGVNGPLVILDHVKFPRYAEIVHLTLPDGTKRSGQVLEVSGSKAVVQVFEGTSGID : 106

TcV-ATPaseB :VMRSKCEFTGKVLELGVSEDMLGRVFNGSGIPIDNGPPVLPEQYRDIQGIPINPRARVYPEEMIQTGISSIDVMTSISRGQKIPLFSGAGLPHNEIAAQIVRQAGL : 202
DdV-ATPaseB :---------- : -
ScV-ATPaseB :VKKTTVDFTCESLRIPVSEDMLGRIFDGSGRPIDNGPKVFADDYLDINGSPINPYARIYPEEMISTGVSAIDTMNSIARGQKIPIFSASGLPHNEIAAQICRQAGL : 193
HsV-ATPaseB1:ARKTTCEFTGDILRTPVSEDMLGRVFNGSGKPIDKGPVVMAEDFLDINGQPINPHSRIYPEEMIQTGISPIDVMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGL : 206
HsV-ATPaseB2:AKKTSCEFTGDILRTPVSEDMLGRVFNGSGKPIDRGPVVLAEDFLDIMGQPINPQCRIYPEEMIQTGISAIDGMNSIARGQKIPIFSAAGLPHNEIAAQICRQAGL : 212

TcV-ATPaseB :VRK-----EGKQEDFCIVFAAMGVNQETARFFRTEFEENGSMEKTVLFMNLANDPTIERIVTPRLALTTAEYLAYDCGKHVLVILTDMSSYADALREVSAAREEVP : 303
DdV-ATPaseB :----------VNMETASFFKRDFEESGSMDRTALSLNLADHPTIERIITPRLALTTAEYLAYQCEKHVLVLLTDMSSYADALREVSAAREEVP : 83
ScV-ATPaseB :VFPTKDVHDGHEENFSIVFAAMGVNLETARFFKQDFEENGSLERTSLFLNLANDPTIERIITPRLALTTAEYLAYQTERHVLTILTDMSSYADALREVSAAREEVP : 299
HsV-ATPaseB1:VKKSKAVLDYHDDNFAIVFAAMGVNMETARFFKSDFEQNGTMGNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVILTDMSSYAEALREVSAAREEVP : 312
HsV-ATPaseB2:VKKSKDVVDYSEENFAIVFAAMGVNMETARFFKSDFEENGSMDNVCLFLNLANDPTIERIITPRLALTTAEFLAYQCEKHVLVILTDMSSYAEALREVSAAREEVP : 318

TcV-ATPaseB :GRRGFPGYMYTDLATIYERAGRVLGRAGSITQIPILTMPNDDITHPIPDLTGYITEGQIYVDRQLHNRQLYPPINILPSLSRLMKNAIGEGMTRKDHGGVSNQMYA : 409
DdV-ATPaseB :GRRRYPGYMYTDLSTIYERAGRIQGRKGSITQIPIVTMPNDDITHPIPDLTGYITEGQIFIDRQLNNRQIYPPINVLPSFSRLMKSAIGEDMTRGDHSEVSNQMYA : 189
ScV-ATPaseB :GRRGYPGYMYTDLSTIYERAGRVEGRNGSITQIPILTMPNDDITHPIPDLTGYITEGQIFVDRQLHNKGIYPPINVLPSLSRLMKSAIGEGMTRKDHGDVSNQLYA : 405
HsV-ATPaseB1:GRRGFPGYMYTDLATIYERAGRVEGRGGSITQIPILTMPNDDITHPIPDLTGFITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHGDVSNQLYA : 418
HsV-ATPaseB2:GRRGFPGYMYTDLATIYERAGRVEGRNGSITQIPILTMPNDDITHPIPDLTGYITEGQIYVDRQLHNRQIYPPINVLPSLSRLMKSAIGEGMTRKDHADVSNQLYA : 424

TcV-ATPaseB :AYAISRDILAMKAVVGEEALSSEDLLHLEFLEKFEKKFICQGFYESRDVFQSLDLGWQLLRTFPKVLLMKIDVKTRNEFYDRQPERK---------- : 496
DdV-ATPaseB :NYAIGKDVQAMKAVVGEEALSSEDKLYLEFLERFESSFVGQNHYENRDIFNSLDLGWSLLKTFPSNLLKRITEKTIKQYYSRSSKGT---------- : 276
ScV-ATPaseB :KYAIGKDAAAMKAVVGEEALSIEDKLSLEFLEKFEKTFITQGAYEDRTVFESLDQAWSLLRIYPKEMLNRISPKILDEFYDRARDDADEDEEDPDTRSSGKKKDAS : 511
HsV-ATPaseB1:CYAIGKDVQAMKAVVGEEALTSEDLLYLEFLQKFEKNFINQGPYENRSVFESLDLGWKLLRIFPKEMLKRIPQAVIDEFYSREGALQDLAPDTAL---------- : 513
HsV-ATPaseB2:CYAIGKDVQAMKAVVGEEALTSDDLLYLEFLQKFERNFIAQGPYENRTVFETLDIGWQLLRIFPKEMLKRIPQSTLSEFYPRDSAKH---------- : 511

Figure 5. Gene amplification and construct of TcV-ATPase B. A. The 1491 nucleotides sequence of TcV-ATPase B encoding gene was amplified by *pfu* Ultra High-Fidelity DNA polymerase (Stratagene). B. TcV-ATPase B tagged at the C-terminus with GFP in BamHI and HindII sites of pTEX expression vector (pTEX-TcV-ATPase B-GFP).

Figure 5.

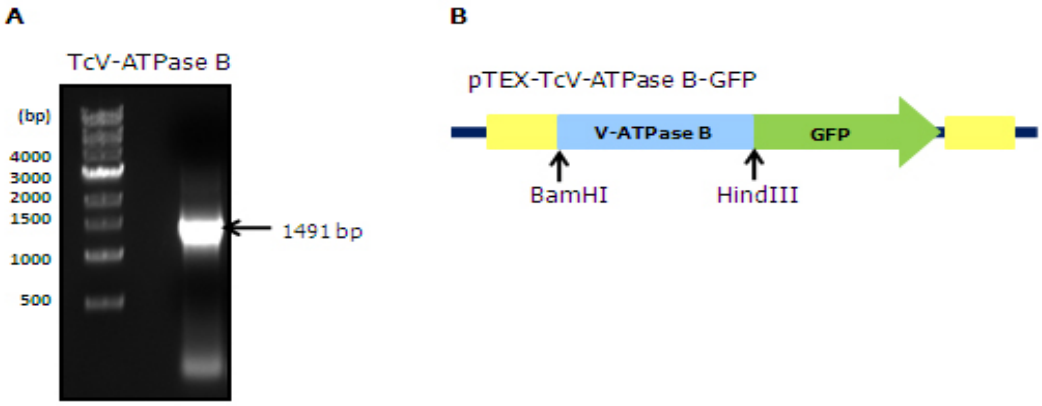

A
TcV-ATPase B
(bp)
4000
3000
2000
1500
1000
500
1491 bp
B
pTEX-TcV-ATPase B-GFP
V-ATPase B
GFP
BamHI
HindIII

Figure 6. Localization of the TcV-ATPase B in the contractile vacuole. A. TcV-ATPase B-GFP localizes to the contractile vacuole bladder. B. Cells under hypo-osmotic condition by exposing them to a 150 mOsm buffer. C. Immunofluorescence assay of TcV-ATPase B using human calmodulin antibody (CaM, red). Green, TcV-ATPase B-GFP. TcV-ATPase B does not co-localize with CaM. Scale bars = 10μm.

Figure 6

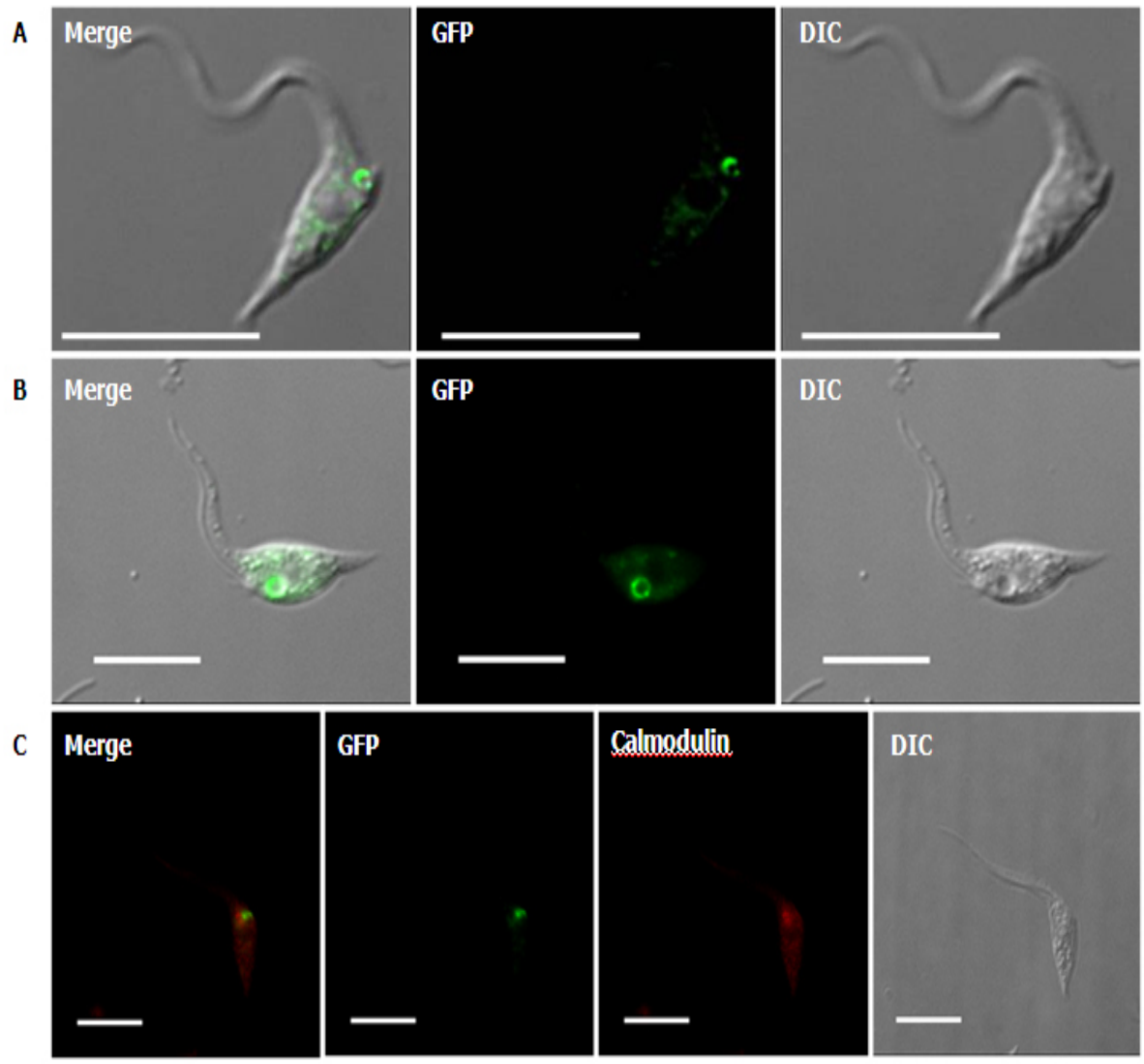


Figure 7. Western blot analysis of TcV-ATPase B-GFP over-expressed in *T. cruzi*. WT *T. cruzi* epimastigote, GFP over-expressing cells and TcV-ATPase B over-expressing cells were extracted, separated between soluble and pellet fractions, loaded by SDS-PAGE (30 μg of protein extracts respectively), and processed for immunoblotting with polyclonal GFP Ab (Molecular Probes). The 80 kDa V-ATPase B-GFP band was detected in both soluble and membrane fraction of the TcV-ATPase B over-expressing parasites. In WT and TcV-ATPase B epimastigotes, a 45 kDa protein band is found as a cross reacting with GFP Ab was detected. In GFP over-expressing cells showed a 30 kDa band in both soluble and pellet fractions. WT, wild type epimastigote; S, soluble fraction; and P, membrane-associated fraction.

Figure 7.

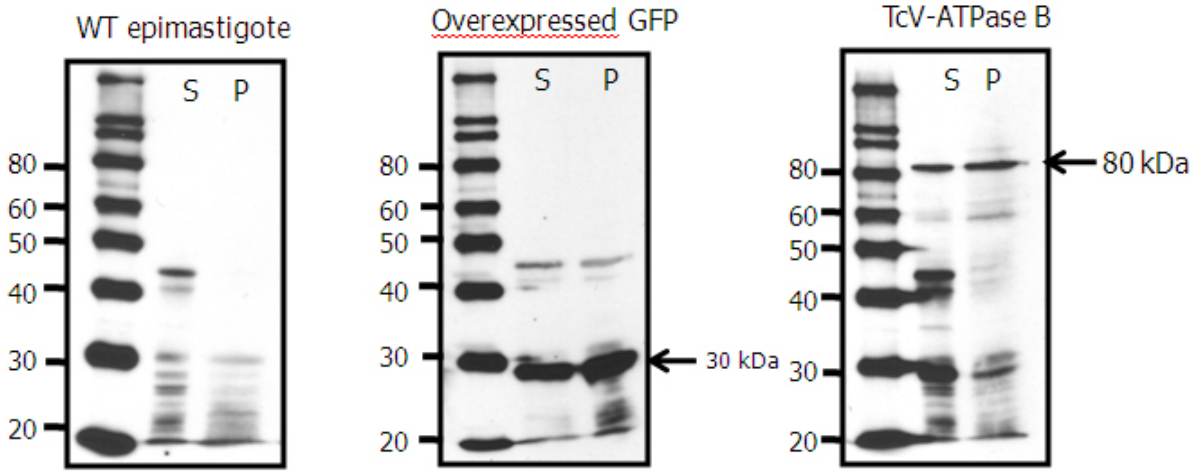
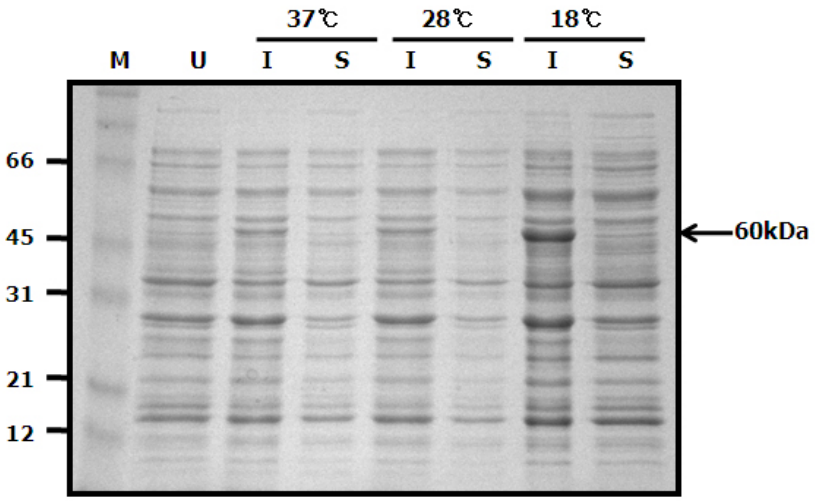

WT epimastigote
S
P
80
60
50
40
30
20
Overexpressed GFP
S
P
80
60
50
40
30
20
30 kDa
TcV-ATPase B
S
P
80
60
50
40
30
20
80 kDa

Figure 8. Induction and purification of recombinant TcV-ATPase B protein A. Expression His-tagged TcV-ATPase B recombinant protein was induced in *E. coli* (BL21 codon plus) with IPTG at 37℃, 28℃, and 18℃. M; Broad range protein marker (Bio-Rad), U; uninduced, I; induced, and S; induced soluble fraction. B. His-tagged TcV-ATPase B recombinant protein was purified from inclusion bodies. The numbers indicates elution fraction.

Figure 8.

A


37℃
28℃
18℃
M
U
I
S
I
S
I
S
66
45
31
21
12
60kDa

B

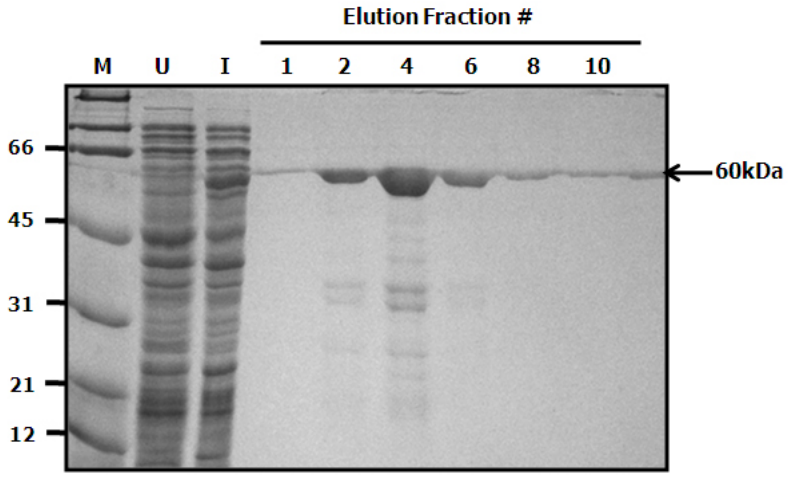

Elution Fraction #
M
U
I
1
2
4
6
8
10
66
45
31
21
12
60kDa

Figure 9. Western blot analysis with polyclonal TcV-ATPase B Ab. A 60 kDa V-ATPase B recombinant protein (5 μg) was only detected. The V-ATPase B protein in both WT epimastigote and TcV-ATPase B over-expressing cells were not detected with the polyclonal α-TcV-ATPase B antibody. Bands of the size between 40 ~ 50 kDa might be cross-reaction of polyclonal α-TcV-ATPase B or backgrounds. M; Broad range protein marker (Bio-Rad), S; soluble fraction, P; membrane fraction, WE; whole cell extract, and R; TcV-ATPase B recombinant protein.

Figure 9.

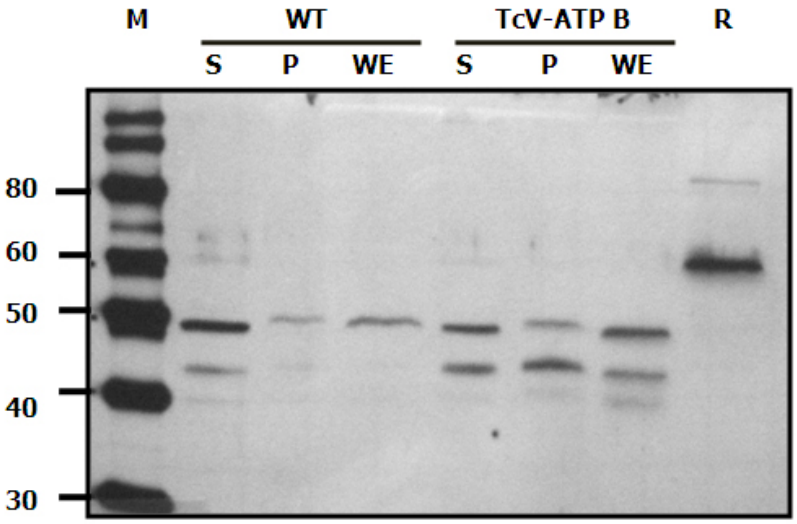
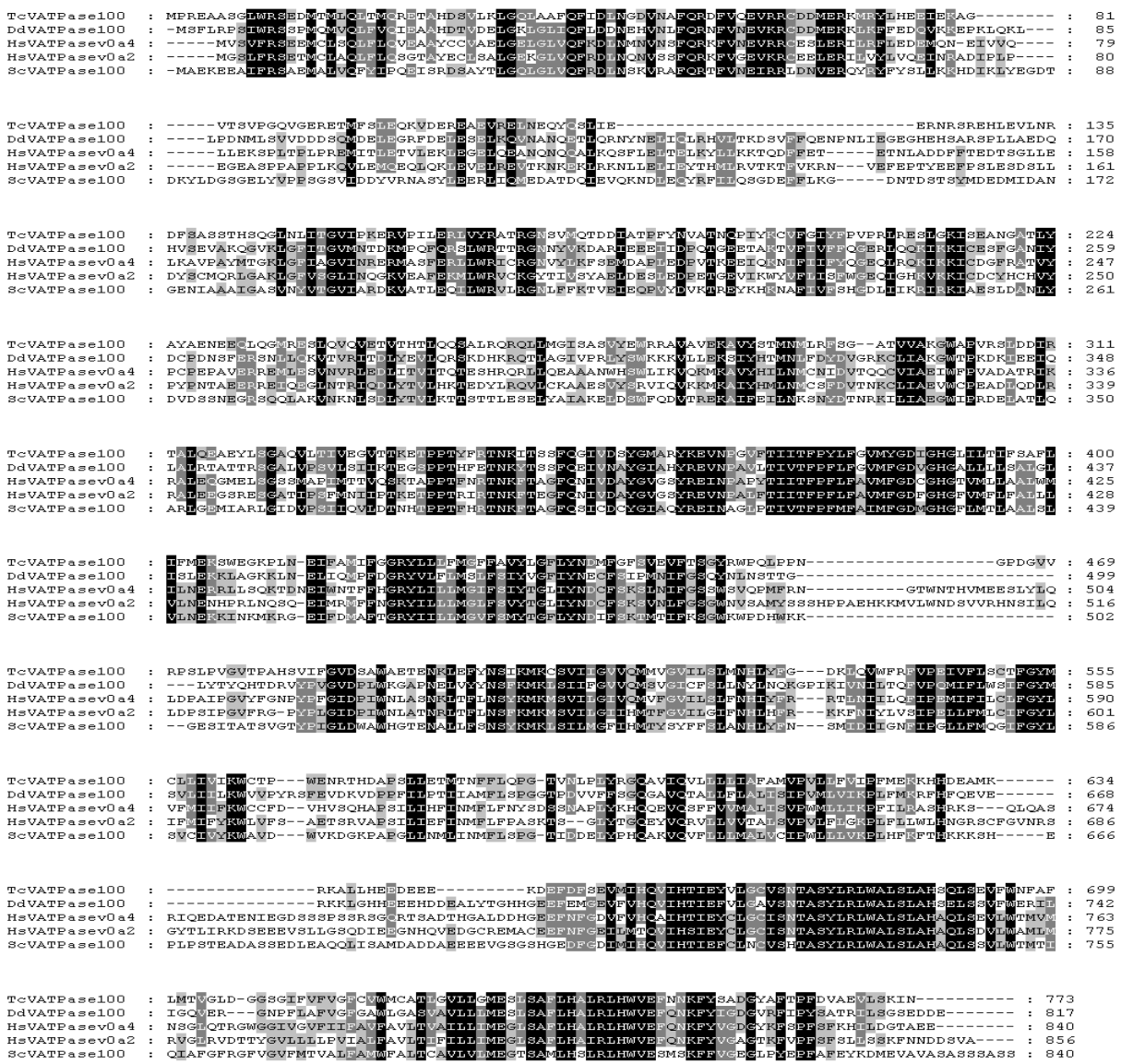


Figure 10. Sequence alignments of TcV-ATPase *a*. Sequence alignment was performed by using Multiple Sequence Alignment by CLUSTALW and GeneDoc. Identical amino acids are shown in black boxes. Conserved changes are shown in light and dark gray boxes. Numbers correspond to amino acid positions in each polypeptide. The amino acid sequence for the *T.cruzi* V-ATPase subunit a, the *Dictyostelium* V-ATPase subunit a (VatM; GenBank ID,DDB0216215), Human V-ATPase subunit a (ATP6V0A2; GenBank ID, 23545 and APT6V0A4; GenBank ID, 50617), and Yeast (VPH1p/STV1p; GenBank ID, 854444) are shown.

Figure 10.


TcVATPase100 : MPREAASGLWRSEDWTMLQLTMQRETAHDSVLKLGQLAAFQFIDLNGDVNAFQRDFVQEVRRCDDMERKMRYLHEEIERAG-------- : 81
DdVATPase100 : -MSFLRPSIWRSSPWCMVQLFVQIEAAHDTVDELGKLGLIQFLDDNEHVNIFQRNFVNEVKRCDDMERKLKFFEDQVKKEPKLQKL--- : 85
HsVATPasev0a4 : -----MVSVFRSEEWCLSQLFLQVEAAYCCVAELGELGLVQFKDLNMNVNSFQRNFVNEVRRCESLERILRFLEDFMQN-EIVVQ---- : 79
HsVATPasev0a2 : -----MGSLFRSETWCLAQLFLQSGTAYECLSALGEKGLVQFRDLNQNVSSFQRNFVGEVKRCEELERILVLVQEINRADIPLP---- : 80
ScVATPase100 : -MAEKEEAIFRSAEWALVCFYIPQCISRDSAYTLGQLGLVQFRDLNSKVRAFQRTFVNEIRRLDNVERQYRYFYSLLKKHDIKIYEGDT : 88

TcVATPase100 : -----VTSVPGQVGERETWFSLFQKVDERAALVRELNEQYCSLIE-------------------------------ERNRSREHLEVLNR : 135
DdVATPase100 : ----LPDNMLSVVDDDSQWDELFGRFDETESETKQVNANQLTLQRNYNEFIQLRHVLTKDSVFQENPNLIEGEGHEHSARSPLLAEDQ : 170
HsVATPasev0a4 : -----LLEKSPLTPLPREWTTLETVLEKIEGETQEANQNQCALKQSFLETTELKYLLKKTQDFFET-----ETNLADDFFTEDTSGLLE : 158
HsVATPasev0a2 : -----EGEASPPAPPLKCVLEMCEQLQKIEVETRETVTKNKCFKLRKNLLEFIEYTHQLRVTKTFVKRN---VEFEPTYEEFPSLESDSLL : 161
ScVATPase100 : DKYLDGSGELYVPPSGSVIDDYVRNASYLEERIIQMEDATDCIEVQRNDLEQYRPIIQSGDELFLKG-----DNTDSTSYMDEDMIDAN : 172

TcVATPase100 : DFSASSTHSQGLNLITGVIPKERVPILERLVYRATRGNSVLQTDDFATPFYNVAINCPIYKCVRGIYFPVPRLRESIGKISEANGATLY : 224
DdVATPase100 : HVSEVAKQGVKLGPIIGVMNTDKMPQFQRSLWFTTRGNNYVKDARFEEEIIDPCAGEETANTVFIVFFQGERIQQKIKKICESFGANIY : 259
HsVATPasev0a4 : LKAVPAYMTGKLGTIAGVINRERMASFERLLWRICRGNVVLKFSPMDAPLEDPVDKEEICNNIFIFYCGEQLRQKIKKICDGFRATVY : 247
HsVATPasev0a2 : DYSCMQRLGARLGTVSGLINQGKVEAFEKMLWRVCKGYTIVSYAPLDESLEDPEDTGEVIKWYVFLISLWGECIGHKVKKICDCYHCHVY : 250
ScVATPase100 : GENIAAAIGASVNMVTGVIARDKVATLEQLLWRVLRGNLFFKTVPLEQPLYDVKIPEYKHKNAFIVFSHGDLIKRIPKKIAESLDFNLY : 261

TcVATPase100 : AYAENEECLQGMRSSLQVCVETVTHTLQQSALRQRQLLMGISASVYEWRRAVAVERAVYSTMNMLRFSG--ATVVAKGWAEVRSLDDIR : 311
DdVATPase100 : DCPDNSFERSNLLCKVTVRITDLVEVLQRSKDHKRQTIAGIVPRLYSWKKKVLLEKSIYHTMNIFDYIVGRKCLIARGWTEKDKLEEIQ : 348
HsVATPasev0a4 : PCPEPAVERRELMLFSVNVRIEDLITVTTCTESHRQRLLQEAAANWHSWLIKVQKMKAVYHILNMCNIIVTQQCVIAEIWFFVADATRIK : 336
HsVATPasev0a2 : PYPNTAEERREIQCQLNTRIQDLYTVLHKTEDVLRQVLCKAAESVYSRVICVKKMKAIYHMLNMCSFLVTNKCLIAEVMCEEADTQDLR : 339
ScVATPase100 : DVDSSNEGRSQQLAKVNKNISDLYTVLKTTSTTLESELYAIAKELDSWFQDVTREKAIEEILNKSNYLTNRKILIAEGWIEPRDELATLQ : 350

TcVATPase100 : TALQEAEYLSCAQVLTIVEGVTTKETPPTYFETNKTISSFQGIVDSYGMARYKEVNPGVFTIITFPYLFGVMYGDIGHGLIITIFSAFL : 400
DdVATPase100 : LALRTATTRSGALVPSVLSIIKTEGSPPTHFETNKYTSSFQEIVNAYGIAHYREVNPAVLTIVTFPFLFGVMFGDVGHGALLLLSALGL : 437
HsVATPasev0a4 : RALECGMELSCSSMAPIMTTVQSKTAPPTFNTTNKFTAGFQNIVDAYGVGSYREINPAPYTIITFPFLFAVMFGDCGHGTVMLLAALWM : 425
HsVATPasev0a2 : RALEEGSREGCATISFMNTIPTKETPPTRITTNKFTEGFQNIVDAYGVGSYREVNPALFTIITFPFLFAVMFGDFGHGFVMFLFALLL : 428
ScVATPase100 : ARLGPMIARLGIDVPSILQVLDTNHTPPTFHETNKFTAGFQSICDCYGIACYREINAGLPTIVTFPFMFAIMFGDMGHGFLMTLAALSL : 439

TcVATPase100 : IFMEKSWEGKPLN-EIFALIFGGRYLLFMCFEAVYLCFLYNDMFGFSVEVFTSGYRWPQLPPN----------GPDGVV : 469
DdVATPase100 : ISLEKKLAGKKLN-ELIQLFFDGRYVLFIMSIFSIYVGFIYNECFSIPMNIFGSCWNLNSTTG---------- : 499
HsVATPasev0a4 : ILNERRLLSQKTDNSIWNTFFHGRYLILLMGIFSIYTGLIYNDCFSKSLNIFGSSWSVQPMFRN----------GTWNTHVMEESLYLQ : 504
HsVATPasev0a2 : VLNENHPRLNQSQ-SIMPTFFNGRYILLLMGLFSVYTGLIYNDCFSKSVNLFGSGWNVSAMYSSSHPPAEHKKMVLWNDSVVRHNSILQ : 516
ScVATPase100 : VLNEKKINKMKRG-EIFDVAFTGRYIILLMGVFSMYTGFLYNDIFSKTMTIFPKGWKWPDHWKK---------- : 502

TcVATPase100 : RPSLPVGVTPAHSVIFGVDSAWAETENKLEFYNSIKMKCSVIIGVVQMMVGVILSLMNHIYFG---DKLQVWFRFVPEIVFLSCTFGYM : 555
DdVATPase100 : ---LYTYQHTDRVYFVGVDPLWKGAPNPLVYYNSFKMKLSIIFGVVQMSVCICFSLLNYLNQKGPIKIVNILTQFVPQMIFLWSIFGYM : 585
HsVATPasev0a4 : LDPAIPGVYFGNPYFFGIDPIWNLASNKLTFLNSYKMKMSVILGIVQMVFGVILSLFNHIYFR---RTINILQFIPEMIFTLCIFGYL : 590
HsVATPasev0a2 : LDPSIPGVFRG-PYPLGIDPIWNLATNRLTFLNSFKMKMSVILGIIHMTFGVILGIFNHLHFR---KKFNIYLVSIPELLFMLCIFGYL : 601
ScVATPase100 : --GESITATSVGTYPIGLDWAWHGTENALLFSNSYKMKLSILMGFIHMTYSYFFSLANHLYFN---SMIDIIGNFIPGLLFMQGIFGYL : 586

TcVATPase100 : CLLIVIKWCTP---WENRTHDAPSLLETMTNFFLQPG-TVNLPLYRGCAVIQVLLLLIAFAMVPVLLFVIPFMEKKHHDEAMK------ : 634
DdVATPase100 : SVLIILKWVVPYRSPEVDKVDPPFILPTIIAMFLSPGGTPLVVFFSGCGAVCTALLFLALISIPVMLVIKPLFMKRPHFQEVE------ : 668
HsVATPasev0a4 : VFMILPKWCCFD--VHVSQHAPSILIHFINMFLFNYSDSSWAPTYKHQPWCSFFVVMALISVPWMLLIKPFILRASHRKS---QLQAS : 674
HsVATPasev0a2 : IFMIFYKWLVFS--AETSRVAPSILIEFINMFLFPASKTS--GIYTGCEYVCRVLLVVTALSVEVLFLGKFLFLLWLHNGRSCFGVNRS : 686
ScVATPase100 : SVCIVYKWAVD---WVKDGKPAPGLLNMLINMFLSPG-TIIDETYPHQAKVQVFILLMALVCIEWLLLVKPLHFKFTHKKKSH-----E : 666

TcVATPase100 : ----------------RKALLHEDEEE---------KDEFDFSEVMIHQVIHTIEYVLGCVSNTASYLRLWALSLAHSQLSEVFWNFAF : 699
DdVATPase100 : ----------------RKKLGHHGEEHDDEALYTGHHGEEFEMGEVFVHQVIHTIEFVLGAVSNTASYLRLWALSLAHSELSSVFWERIL : 742
HsVATPasev0a4 : RIQEDATENIEGDSSSPSSRSGCRTSADTHGALDDHGEEFNFGDVFVHQATHTIEYCLGCISNTASYLRLWALSLAHAQLSEVLWTMVM : 763
HsVATPasev0a2 : GYTLIRKDSEEEVSLLGSQDIEGNHQVEDGCREMACEEFNFGEIFMTQVIHSIEYCLGCISNTASYLRLWALSLAHAQLSDVLWAMLM : 775
ScVATPase100 : PLPSTEADASSEDLEAQQLISAMDADDAEEEEVGSGSHGEDFGDIMIHQVIHTIEFCLNCVSHTASYLRLWALSLAHAQLSSVLWTMTI : 755

TcVATPase100 : LMTVGLD-GGSGIFVFVGICWMCATLGVLLGMESLSAFLHALRLHWVEFNNKFYSADGYAFTPFDVAEVLSKIN---------- : 773
DdVATPase100 : IGQVER---GNPFLAFVGPGAWLGASVAVLLLMESLSAFLHALRLHWVEFQNKFYTGDGVRFTPYSATRILSGSEDDE------- : 817
HsVATPasev0a4 : NSGLQTRGWGGIVGVFIIFAVFAVLTVAILLIMEGLSAFLHALRLHWVEFQNKFYVGDGYKFSPPSFKHILDGTAEE-------- : 840
HsVATPasev0a2 : RVGLRVDTTYGVLLLPVIALFAVLTIFLLIMEGLSAFLHAIRLHWVEFQNKFYVCAGTKFVPPSFSLLSEKFNNDDSVA---- : 856
ScVATPase100 : QIAFGFRGFVGVFMTVALFAMWFALTCAVLVLMEGTSAMLHSLRLHWVESMSKFFVGEGLPYEPFAFEYKDMEVAVASASSSASS : 840

Figure 11. Gene amplifications and constructs of TcV-ATPase *a*. A. The 2322 nucleotides sequence of TcV-ATPase *a* encoding gene (Tc00.1047053509601.70) was amplified by *pfu* Ultra High-Fidelity DNA polymerase (Stratagene). B. TcV-ATPase *a* tagged at the C-terminus with GFP in BamHI and HindIII sites of pTEX expression vector (pTEX-TcV-ATPase *a*-GFP).

Figure 11.

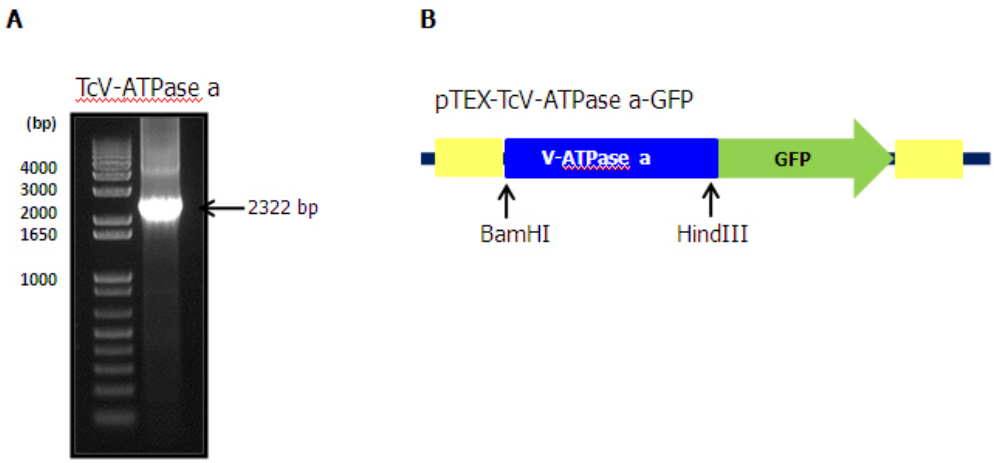

A
TcV-ATPase a
(bp)
4000
3000
2000
1650
1000
2322 bp
B
pTEX-TcV-ATPase a-GFP
V-ATPase a
GFP
BamHI
HindIII

Figure 12. Localization of the TcV-ATPase *a* in the contractile vacuole. A. Fluorescence of TcV-ATPase *a*-GFP distributes in the contractile vacuole. B. Cells under hypo-osmotic conditions by exposing them to an 150 mOsm buffer. The fluorescence localizes in the bladder membrane. Black arrow indicates enlarged bladder. C. Immunofluorescence assay of TcV-ATPase *a* using human calmodulin Ab (Red). Green shows TcV-ATPase *a*-GFP. TcV-ATPase *a*-GFP does not co-localize with calmodulin. Scale bars = 10μm.

Figure 12.

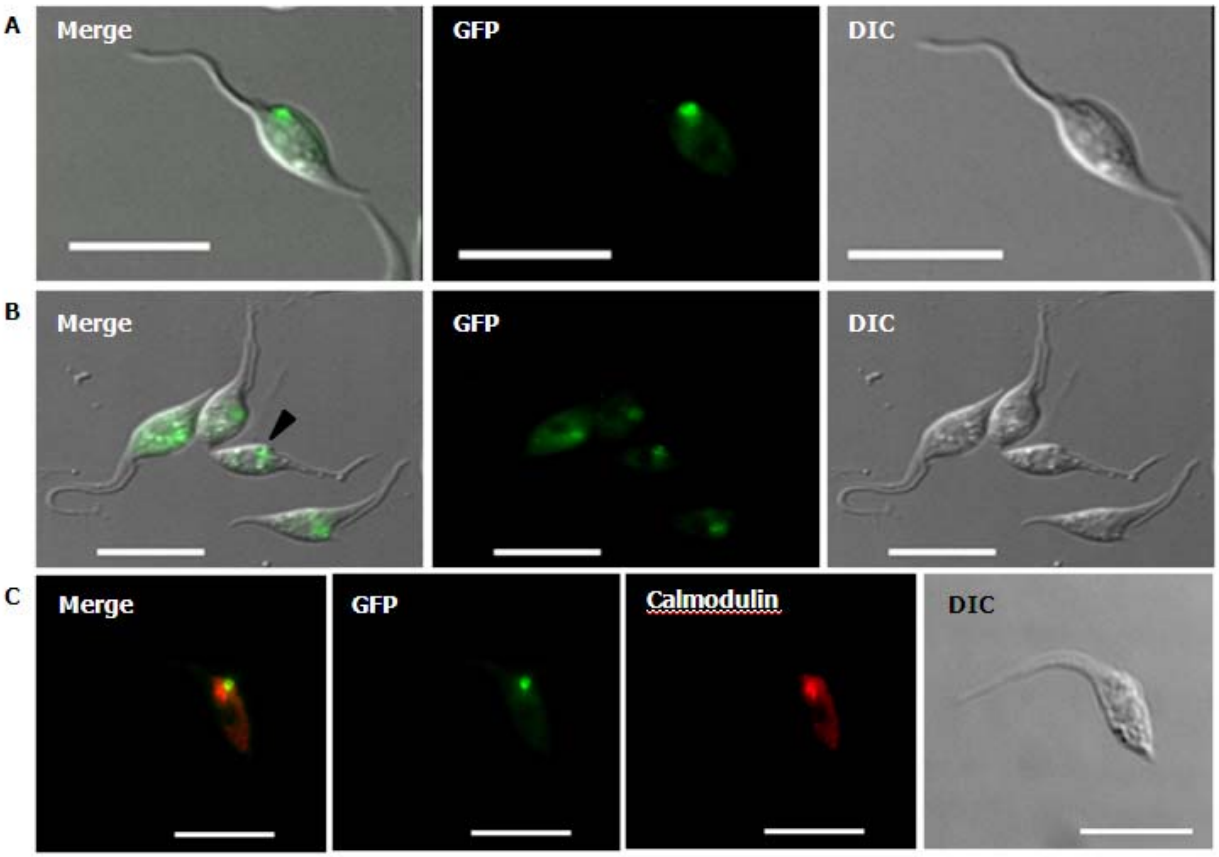


Figure 13. Western blot analysis of TcV-ATPase *a*-GFP over-expressed in *T. cruzi*. WT *T. cruzi* epimastigote and TcV-ATPase *a* cells were extracted, separated between soluble and pellet fractions, loaded by SDS-PAGE (40 μg of protein extracts respectively), and processed for immunoblotting with polyclonal GFP Ab (Molecular Probes). A 110 kDa V-ATPase *a*-GFP was detected in membrane fraction of the TcV-ATPase *a*. S, soluble fraction; and P, membrane-associated fraction.

Figure 13.

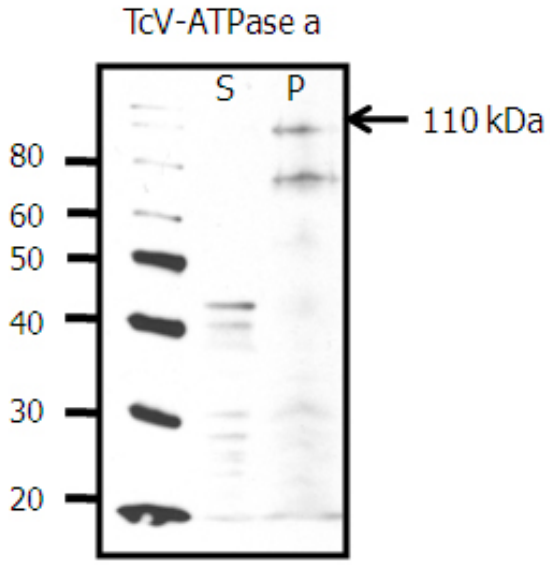


Figure 14. Multiple sequence alignments of R-SNAREs. The sequence analysis was restricted to 15 layers (blue), including 7 layers upstream and 8 layers downstream of the ionic layer (layer '0') and transmembrane domain. The numbers (-7 to 8) on the top of layers show leucine-zipper geometry and high conserved amino acid compositions which form α-helical complex between SNARE proteins. Conserved residues are shaded in light blue. The conserved arginine residues forming the '0'-layer is indicated in orange, and non-conserved '0'-layer is in black. Sequence alignment was performed by using Multiple Sequence Alignment by CLUSTALW and GeneDoc. GeneBank accession numbers for the synaptobrevin vamp family (R-SNAREs) are TcSNARE2.1, TC, Tc00.1047053507625.183; TcSNARE2.2, TC, Tc00.1047053506715.50; sb2, RN, M24105; cbycellubrevin, RN, S63830; sb1 CE AF003281; sec22b, MM, U91538; Snc1, SC, M91157; sb7, MM, X96737; and tomosyn, RN, U92072; PtSyb1-1, PT, AJ566298;PtSyb1-2, PT, CR855907; PtSyb2-1, PT, AJ566299; PtSyb2-2, PT, AJ566300; PtSyb3-1, PT, AJ566301; PtSyb6-1, PT, CR855902; PtSyb7-1, PT, CR855901; PtSyb7-2, PT, CR855900; PtSyb8-1, PT, CR855899; PtSyb9-1, PT, CR855898; PtSyb9-2, PT, CR855897.

Figure 14.

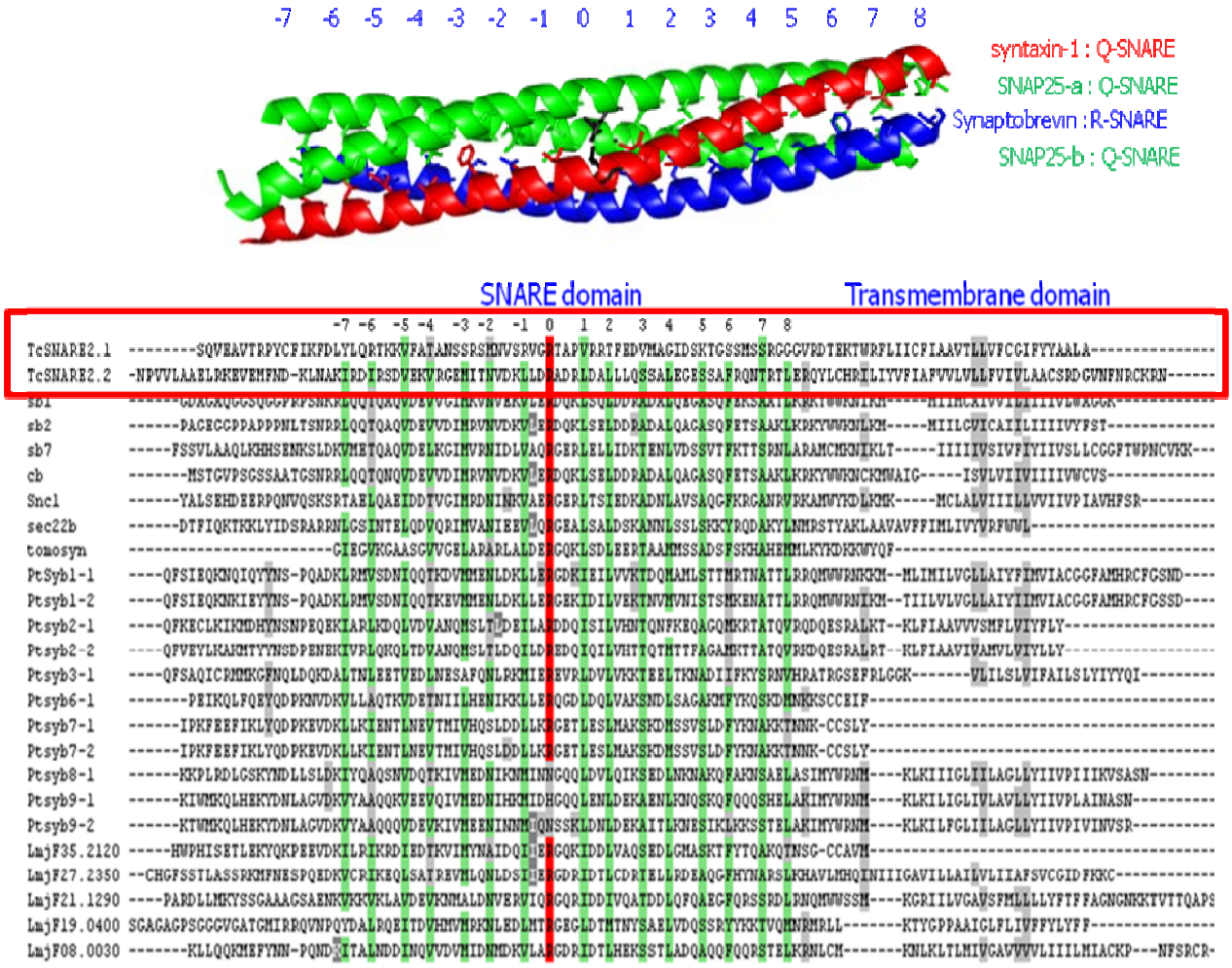

-7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8
syntaxin-1 : Q-SNARE
SNAP25-a : Q-SNARE
Synaptobrevin : R-SNARE
SNAP25-b : Q-SNARE
SNARE domain
Transmembrane domain
TcSNARE2.1
TcSNARE2.2
sb1
sb2
sb7
cb
Sncl
sec22b
tomosyn
PtSyb1-1
Ptsyb1-2
Ptsyb2-1
Ptsyb2-2
Ptsyb3-1
Ptsyb6-1
Ptsyb7-1
Ptsyb7-2
Ptsyb8-1
Ptsyb9-1
Ptsyb9-2
LmjF35.2120
LmjF27.2350
LmjF21.1290
LmjF19.0400
LmjF08.0030

Figure 15. Gene amplifications and constructs of TcSNARE2.1 and TcSNARE2.2. A. The 639 nucleotide sequence of TcSNARE2.1 encoding gene (Tc00.1047053507625.183) and The 648 nucleotide sequence of TcSNARE2.2 encoding gene (Tc00.1047053506715.50) were amplified by *pfu* Ultra High-Fidelity DNA polymerase (Stratagene). B. TcSNARE2.1 and TcSNARE2.2 tagged at the C-terminus with GFP in BamHI and HindII sites of pTEX expression vector (pTEX-TcSNARE2.1-GFP and TcSNARE2.2-GFP).

Figure 15.

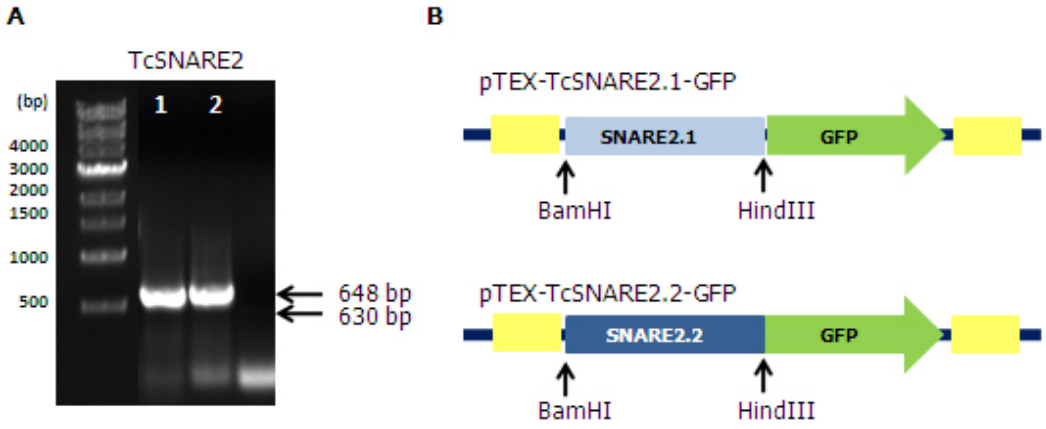

A
TcSNARE2
(bp)
1
2
4000
3000
2000
1500
1000
500
648 bp
630 bp
B
pTEX-TcSNARE2.1-GFP
SNARE2.1
GFP
BamHI
HindIII
pTEX-TcSNARE2.2-GFP
SNARE2.2
GFP
BamHI
HindIII

Figure 16. Localization of the TcSNARE2.1 in the contractile vacuole. A. Fluorescence of TcSNARE2.1-GFP distributes in contractile vacuole. B. The fluorescence localizes in the vicinity of contractile vacuole bladder. Cell under hypo-osmotic conditions by exposing them to an 150 mOsm buffer. C. Immunofluorescence assay of TcSNARE2.1 using anti-human calmodulin antibody (CaM, red) in the spongiome of the contractile vacuole. Green indicates fluorescence signal of TcSNARE2.1-GFP. Yellow indicates the co-localization between TcSNARE2.1 and CaM. Scale bars = 10μm.

Figure 16.

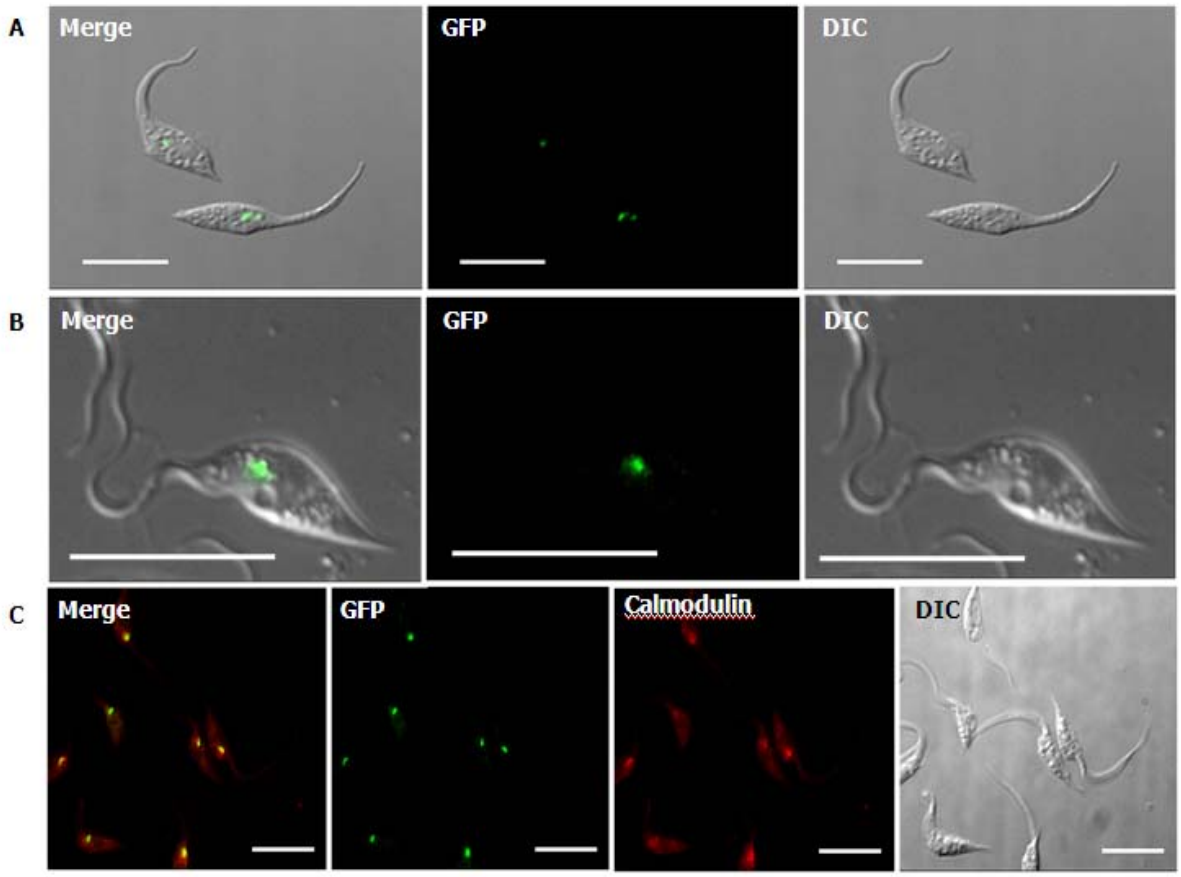


Figure 17. Localization of the TcSNARE2.2 in the contractile vacuole. A. Fluorescence of TcSNARE2.2-GFP distributes in contractile vacuole. B. The fluorescence localizes in the vicinity of contractile vacuole bladder. Cell under hypo-osmotic conditions by exposing them to an 150 mOsm buffer. C. Immunofluorescence assay of TcSNARE2.2 using anti-human calmodulin antibody (CaM, red) in the spongiome of the contractile vacuole. Green indicates fluorescence signal of TcSNARE2.2-GFP. Yellow indicates the co-localization between TcSNARE2.2 and CaM. Scale bars = 10 μm.

Figure 17.

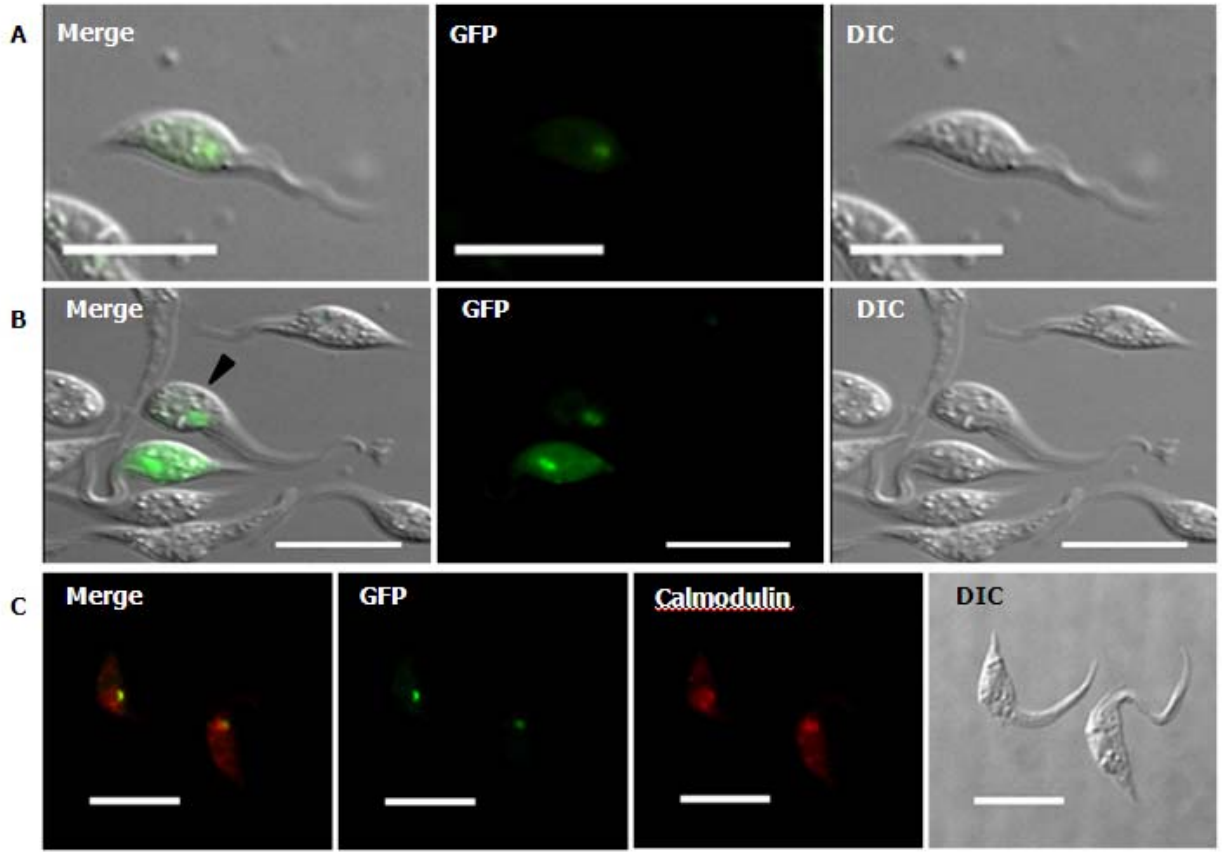


Figure 18. Western blot analysis of TcSNARE2.1-GFP and TcSNARE2.2-GFP over-expressed in *T. cruzi*. WT *T. cruzi* epimastigote, TcSNARE2.1-GFP and TcSNARE2.2-GFP cells were extracted, separated between soluble and pellet fractions, loaded by SDS-PAGE (50μg of protein extracts respectively), and processed for immuno blotting with polyclonal α-GFP Ab (Molecular Probes). A 50 kDa TcSNARE2.1-GFP and TcSNARE2.2-GFP were detected in pellet fraction of the over-expressing cells A 30 kDa protein in the soluble fraction may be a degraded GFP protein. In WT epimastigote, a 45 kDa protein band is found as a cross reaction of α-GFP Ab. WT, wild type epimastigote; S, soluble fraction; and P, membrane-associated fraction.

Figure 18.

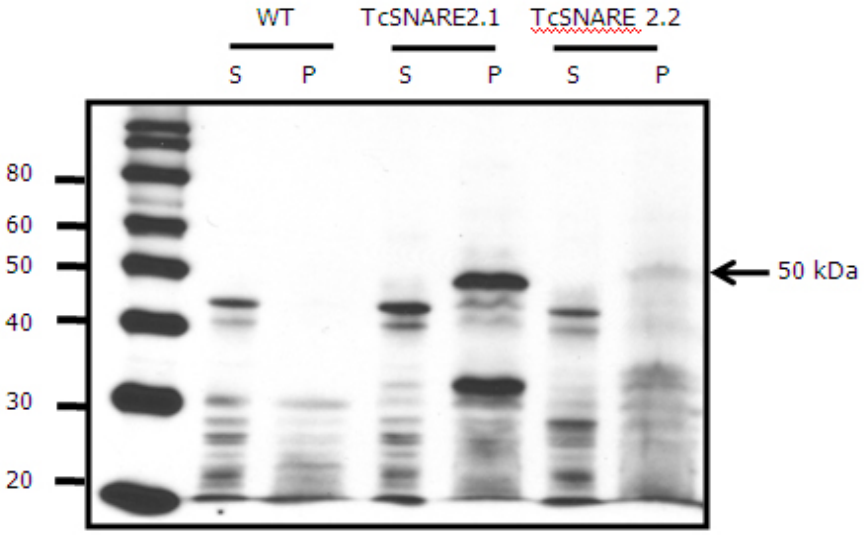
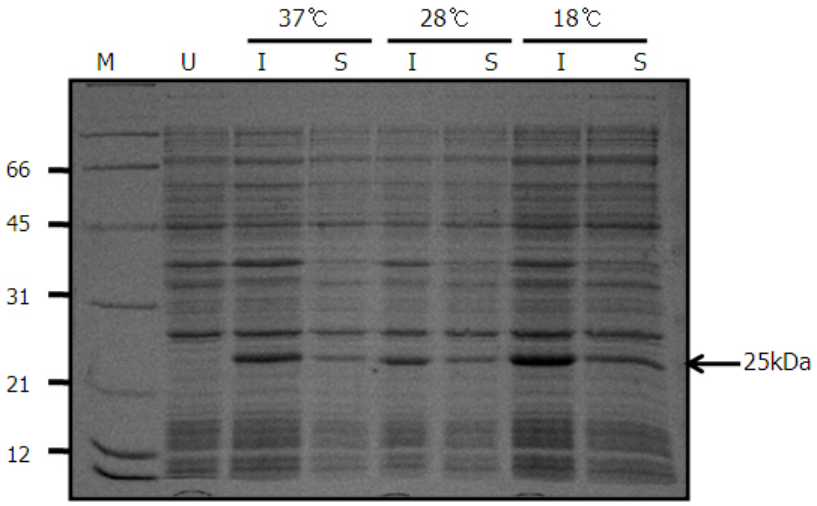


Figure 19. Induction and purification of recombinant TcSNARE2.1 protein A. His-tagged TcSNARE2.1 recombinant protein in *E. coli* (BL21 codon plus) was induced with IPTG at 37C, 28C, and 18C. The sampled were examined by SDS-PAGE. U, uninduced protein extracts; I, induced protein extracts; and S, induced soluble fraction. B. His-tagged SNARE2.1 recombinant protein was purified from the soluble fraction. The numbers indicates elution fraction

Figure 19.

A


37℃
28℃
18℃
M
U
I
S
I
S
I
S
66
45
31
21
12
25kDa

B

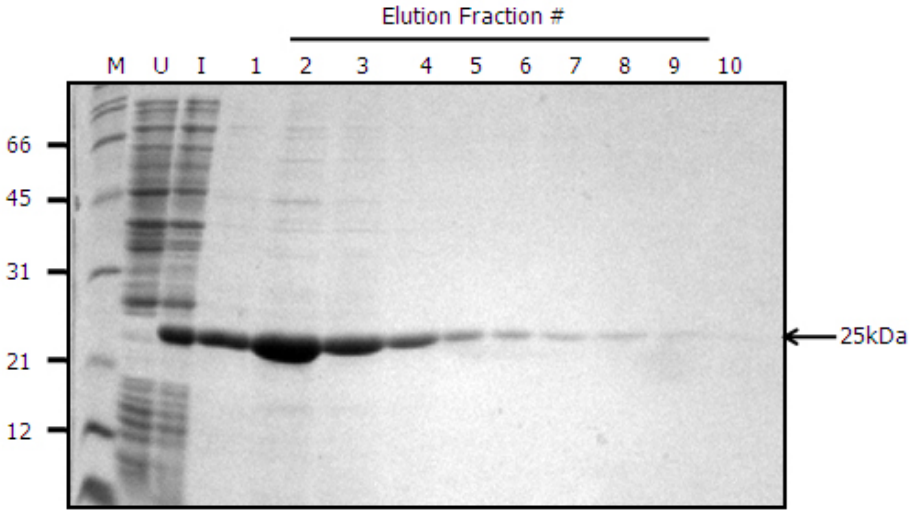

Elution Fraction #
M
U
I
1
2
3
4
5
6
7
8
9
10
66
45
31
21
12
25kDa

Figure 20. Western blot analysis of TcSNARE2.1 over-expressed in *T.cruzi*. WT *T. cruzi* epimastigote and TcSNARE2.1 cells were extracted, separated between soluble and pellet fractions, loaded by SDS-PAGE, and processed for immunoblotting with polyclonal TcSNARE2.1 antibody. A 50 kDa TcSNARE2.1-GFP protein was clearly detected. Approximately a 25 kDa TcSNARE2.1 recombinant protein (5μg) was also detected. A 40 kDa band in the recombinant protein might be cross reaction of polyclonal TcSNARE2.1 antibody against proteins purified in bacterial cells. WT epimastigote was not detected with the anti polyclonal TcSNARE2.1 antibody. WT, wild type epimastigote; S2.1, TcSNARE2.1; GFP, GFP over-expressed cells as a control; S, soluble fraction; and P, membrane-associated fraction; R, recombinant protein of TcSNARE2.1.

Figure 20.

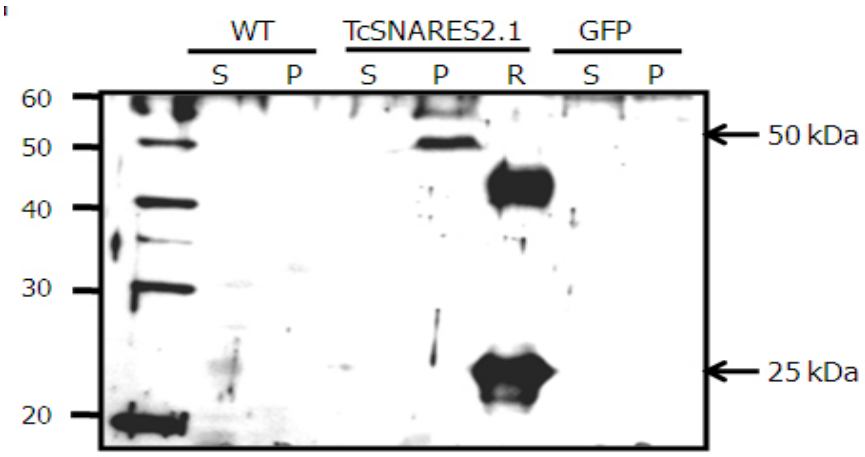


Figure 21. Sequence alignments of TcAP180. TcAP180 belongs to the AP180 family. All members of the AP180 family have an N-terminal ANTH domain that confers binding to phospholipids at the plasma membrane. Alignment was performed using ClustalW. Dashed box indicates ANTH domain. Black underline is conserved ANTH domain consensus sequence *(K/G)A(T/I)xxxxxx(P/L/V)KxK(H/Y).* Identical amino acids are shown in black boxes. Conserved changes are shown in light and dark gray boxes. Numbers correspond to amino acid positions in each polypeptide. The amino acid sequence for the *T.cruzi* AP180, the *Dictyostelium* AP180 (Clma, GenBank ID: DDB0235311), *S. cerevisiae* AP180 (YAP1801, GenBank ID: 856566), and Human AP180 (SNAP91, GenBank ID: 9892) are shown.

Figure 21.

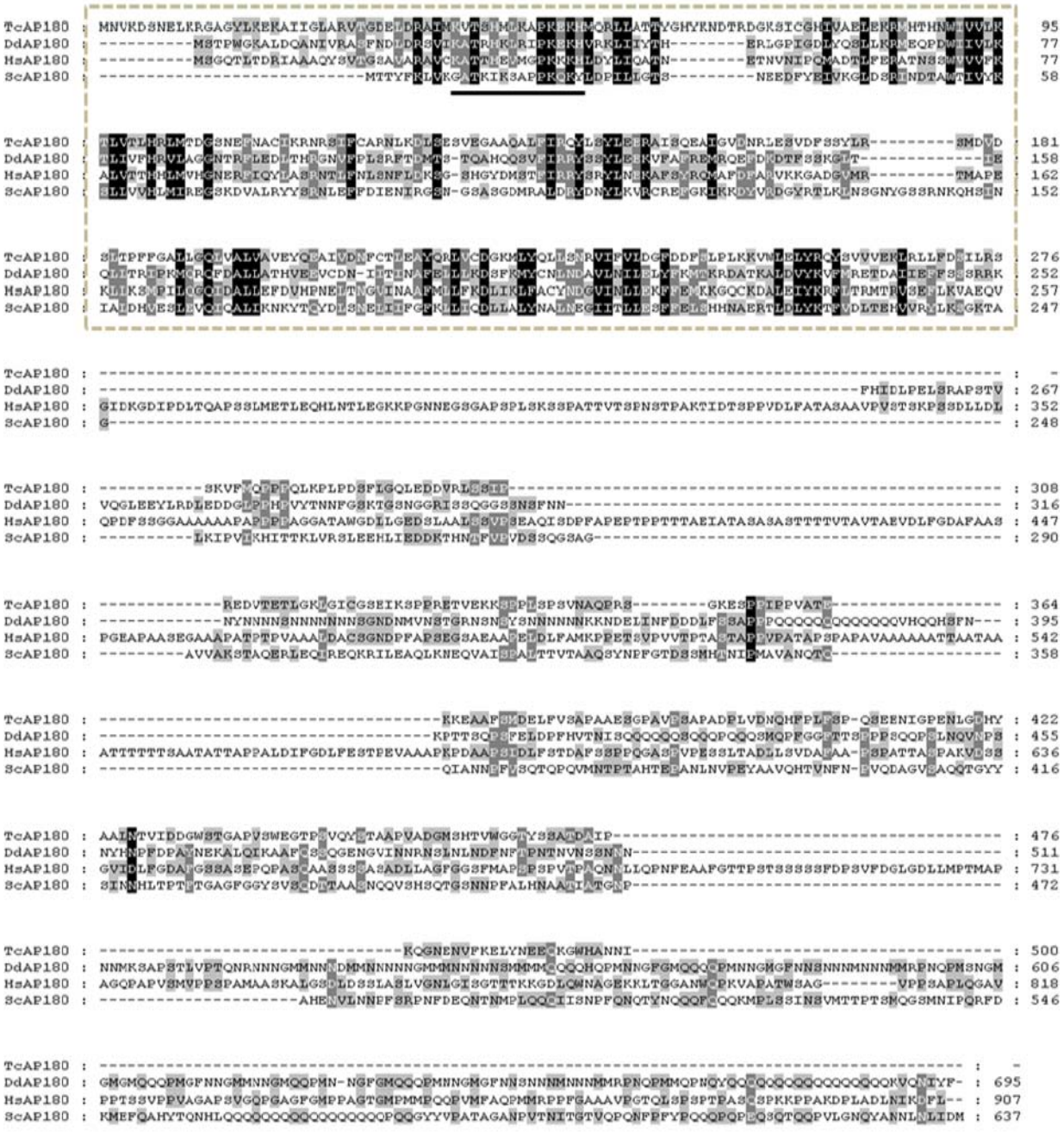


Figure 22. Gene amplification and construct of TcAP180. A. The 1503 nucleotide sequence of TcAP180-encoding gene was amplified by *pfu* Ultra High-Fidelity DNA polymerase (Stratagene). B. TcAP180 tagged at the N-terminus with GFP in BamHI and XhoI sites of pTEX expression vector (pTEX-GFP-AP180).

Figure 22.

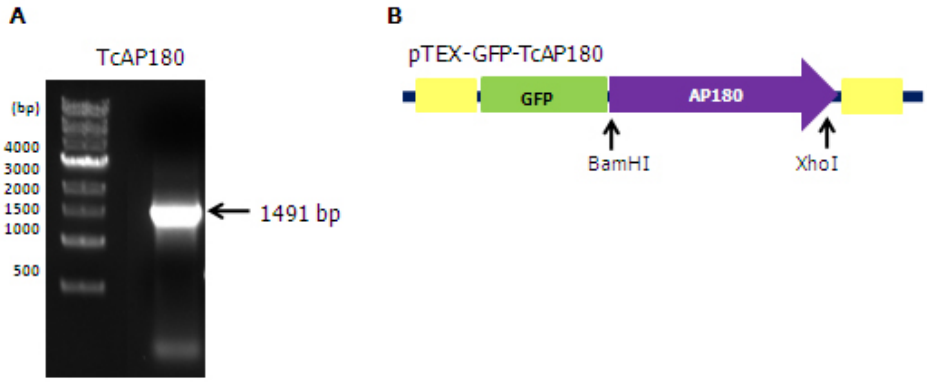

A
TcAP180
(bp)
4000
3000
2000
1500
1000
500
1491 bp
B
pTEX-GFP-TcAP180
GFP
AP180
BamHI
XhoI

Figure 23. Localization of the TcAP180 in the contractile vacuole. A. GFP-TcAP180 locates to the contractile vacuole bladder. B. Cells under hypo-osmotic condition by exposing them to an 150 mOsm buffer. C. Immuno-fluorescence assay of TcAP180 using human calmodulin antibody (red). Green, GFP-TcAP180. TcAP180 does not co-localize with CaM. Scale bars = 10μm.

Figure 23.

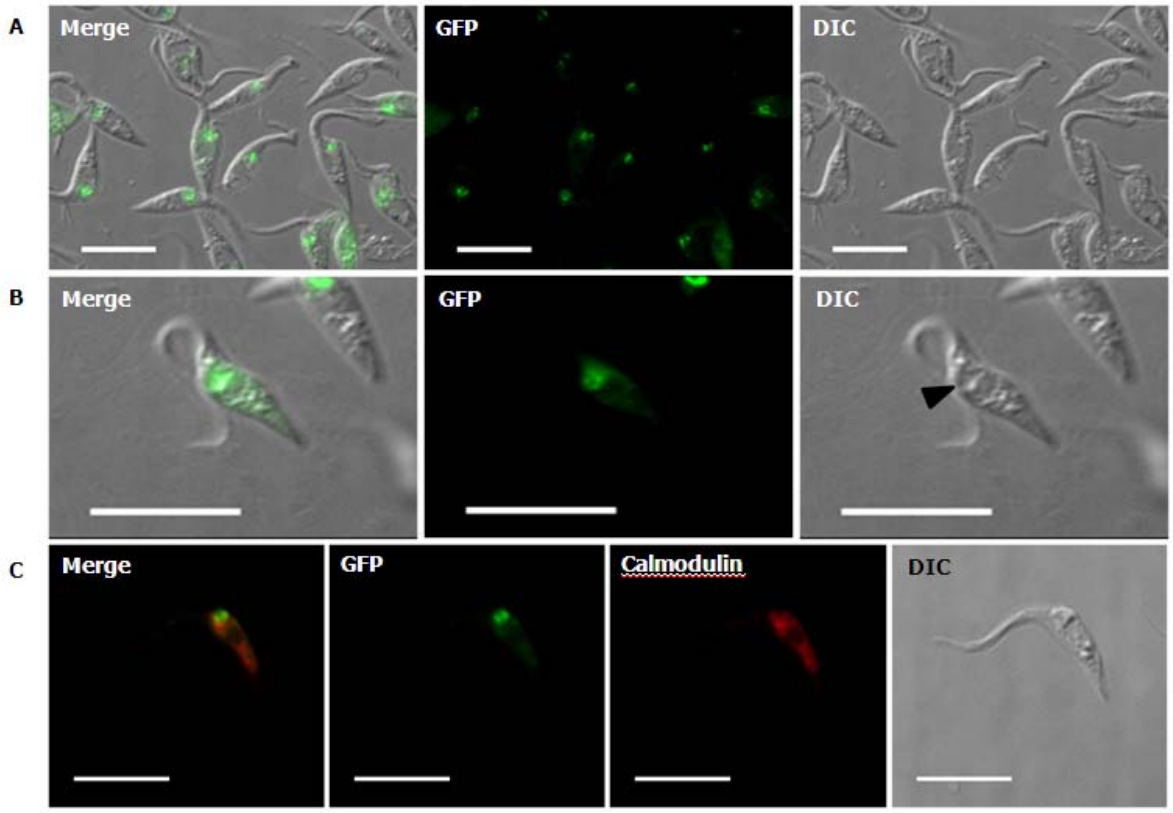


Figure 24. Western blot anaysis of TcAP180 over-expressed in *T. cruzi*. Wide type *T. cruzi* epimastigote and GFP-TcAP180 cells were extracted, separated between soluble and pellet fractions, loaded by SDS-PAGE (40μg of protein extracts respectively), and processed for immunoblotting with polyclonal GFP Ab (Molecular Probes). An 80 kDa GFP-TcAP180 was detected in both soluble and membrane fractions of the over-expressed cells. A 30 kDa protein in the soluble fraction may be degraded GFP protein. In WT epimastigote, a 45 kDa protein band is found as a cross reaction of α-GFP Ab. WT, wild type epimastigote; S, soluble fraction; and P, membrane-associated fraction.

Figure 24.

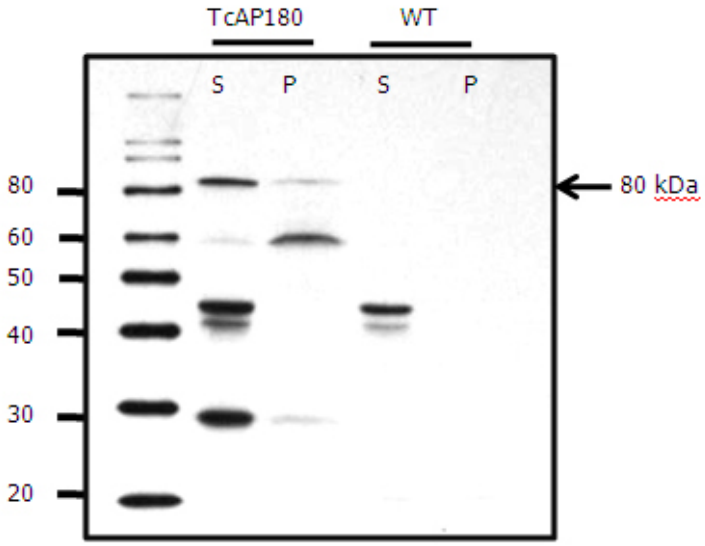


Figure 25. Sequence alignments of TcDisgorgin/Drainin. Sequence alignment was performed by using Multiple Sequence Alignment by CLUSTALW and GeneDoc. GenBank accession numbers for the DdDrainin is AAD00520; DdDisgorgin is DDB0218275; TcDisgorgin/Drainin is Tc00.1047053508723.80. The two conserved catalytic arginines and glutamines for GAP activity are marked with Red boxes.

Figure 25.

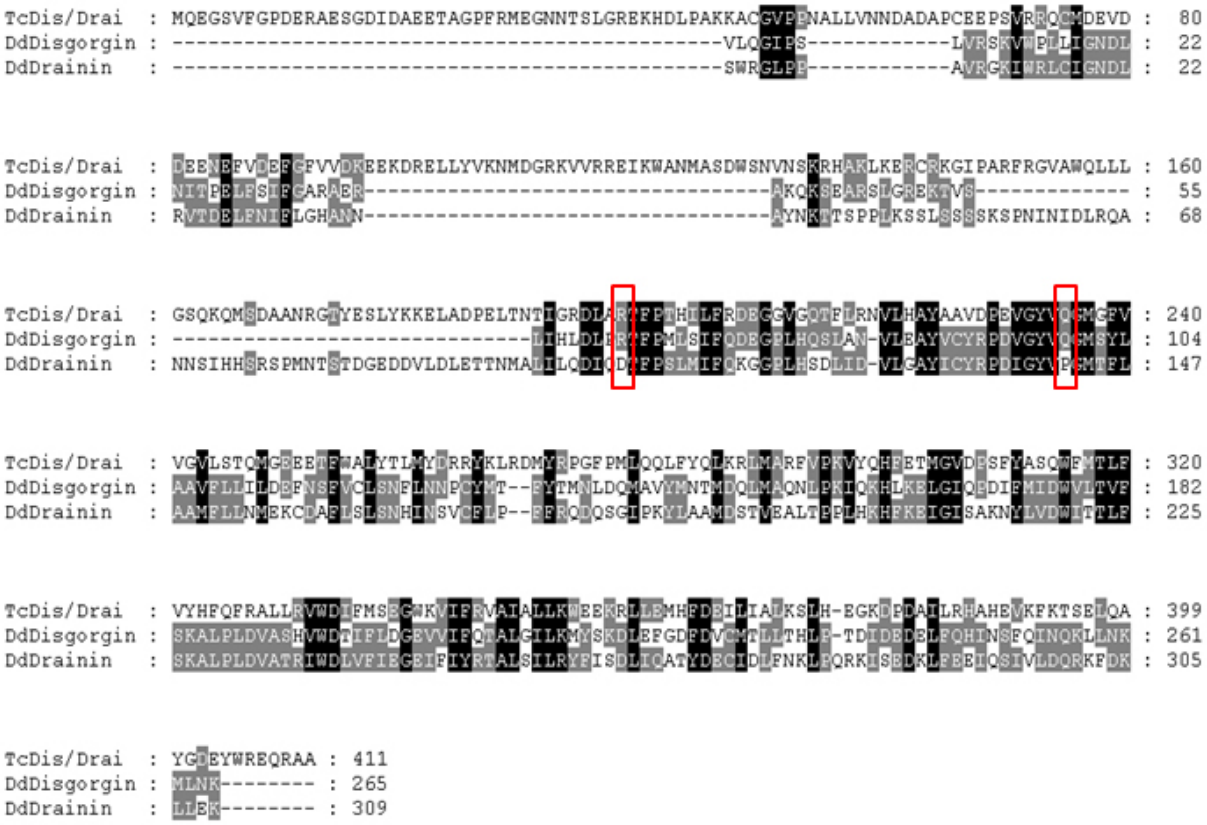

TcDis/Drai : MQEGSVFGPDERAESGDIDAEETAGPFRMEGNNTSLGREKHDLPAKKACGVPENALLVNNDADAPCEEPSVRRQCMDEVD : 80
DdDisgorgin : ---------- : 22
DdDrainin : ---------- : 22
TcDis/Drai : DEENEFVDEGFVVDREEKDRELLYVKNMDGRKVVRREIKWANMASDWSNVNSKRHAKLKERCRKGIPARFRGVAWQLLL : 160
DdDisgorgin : NITPELFSIFGARAER---------- : 55
DdDrainin : RVTDELFNIELGHANN---------- : 68
TcDis/Drai : GSQKQMSDAANRGTYESLYKKELADPELTNTLGRDLARTFPTHLLERDEGGVGQTFLRNVLHAYAAVDPEVGYVQGMGFV : 240
DdDisgorgin : ---------- : 104
DdDrainin : NNSIHHSRSPMNTSTDGEDDVLDLETTNMALLLQDIQDTFPSLMIFQKGGPLHSDLID-VLCAYICYRPDIGYVPGMTFL : 147
TcDis/Drai : VGVLSTQMGEEETFWALYTLMYLRRYKLRDMYRPGFPMLQQLFYQLKRLMARFVPKVYQHEETMGVDESFYASQWFMTLF : 320
DdDisgorgin : AAVFLLILDEFNSFVCLSNFLNNPGYMT--EYTMNLDQMAVYNTMDQLMAQNLPKIQKHLKELGIQEDIFMIDWVLTVF : 182
DdDrainin : AAMFLLNMEKCDAFLSLSNHINSVGFLP--EFRQDQSGIPKYIAAMDSTVEALTPPLHKHFKEIGISAKNYLVDWITTLF : 225
TcDis/Drai : VYHFQFRALLRVWDIFMSEGWKVIFRVAIALLKWEEKRLLEMHFDEILIAVKSLH-EGKDPDAILRHAHEVKFKTSELQA : 399
DdDisgorgin : SKALPLDVASHVWDTIFLDGEVVIFQTALGILKMYSKDLEFGDFDVCMTLLTHLP-TDIDEDELFQHINSFQINQKLLNK : 261
DdDrainin : SKALPLDVATRIWDLVFIEGEIFIYRTALSILRYFISDLIQATYDECIDLFNKLPQRKHSEDKLFEEIQSIVLDQRKFDK : 305
TcDis/Drai : YGEEYWREQRAA : 411
DdDisgorgin : MLNR-------- : 265
DdDrainin : LLEK-------- : 309

Figure 26. Gene amplifications and constructs of TcDisgorgin/Drainin. A. The 1236 nucleotide sequence of TcDisgorgin/Drainin encoding gene (Tc00.1047053508723.80) was amplified by *pfu* Ultra High-Fidenlty DNA polymerase (Stratagene). B. TcDisgorgin/Drainin tagged at the C-terminus with GFP in XbaI and EcoRV sites of pTREX expression vector (pTREX-TcDisgorgin/Drainin-GFP).

Figure 26.

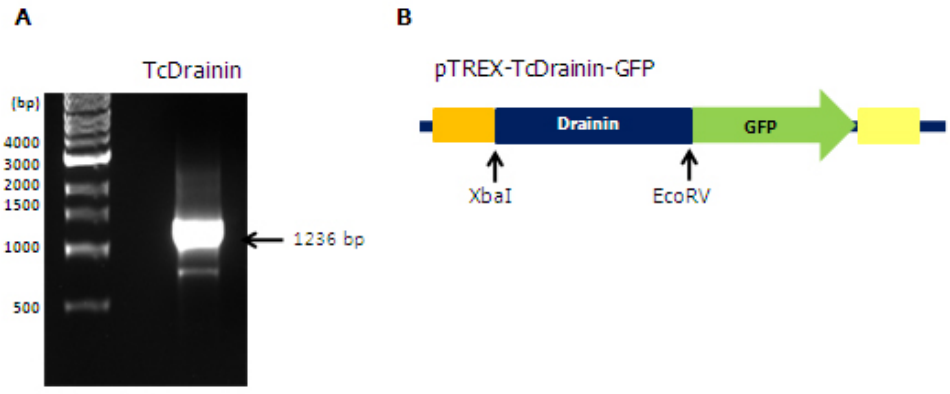

A
TcDrainin
(bp)
4000
3000
2000
1500
1000
500
1236 bp
B
pTREX-TcDrainin-GFP
Drainin
GFP
XbaI
EcoRV

Figure 27. Localization of the TcDisgorgin/Drainin in cytosol and flagella pocket. A. Cells under hypo-osmotic conditions by exposing them to an 150 mOsm buffer. Fluorescence of TcDisgorgin/Drainin-GFP distributes in cytosol and a small proportion concentrates anterior to the kinetoplast in a region near the flagella pocket. TcDisgorgin overexpressed epimastigotes were more sensitive to hypo-osmotic stress than epimastigotes expressing GFP alone under hypo-osmotic buffer (150mOms). B. Immune-fluorescence assay of TcDisgorgin/Drainin using anti-human calmodulin A (CaM, red) as a spongiome maker. But, they did not co-localize. C. Immune-fluorescence assay of TcDisgorgin/Drainin using Concanavalin A (ConA, red) in the flagella pocket. Green indicates fluorescence signal of TcDisgorgin/Drainin-GFP. Yellow indicates the co-localizaion between TcDisgorgin/Drainin and ConA. Blue shows kinetoplast DNA. Scale bars = 10μm.

Figure 27.

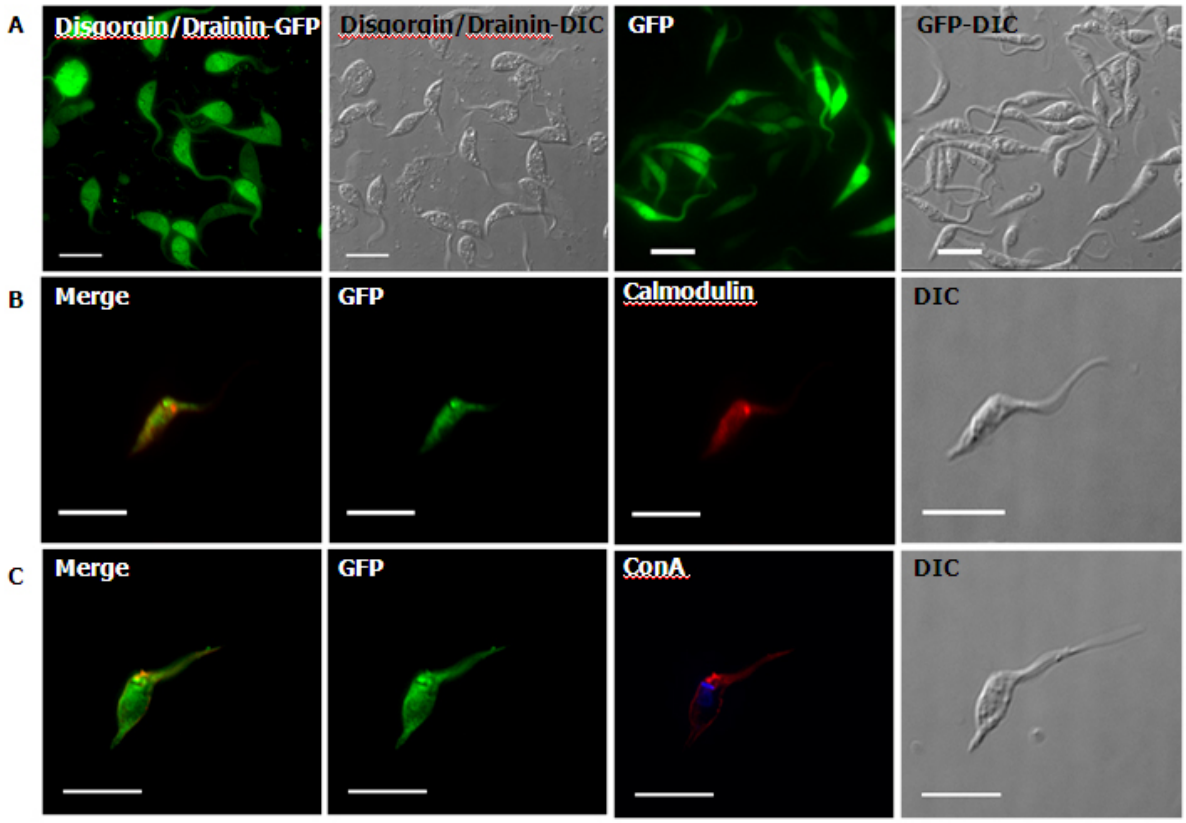
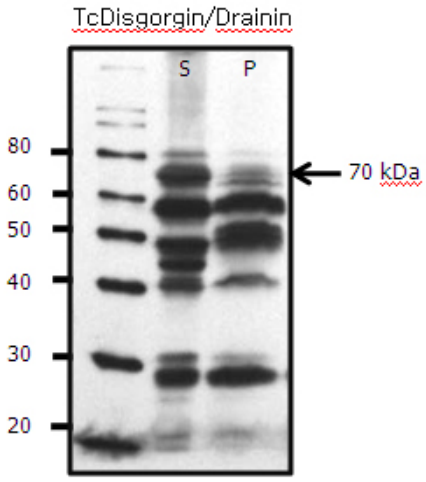

A
Disgorgin/Drainin-GFP
Disgorgin/Drainin-DIC
GFP
GFP-DIC
B
Merge
GFP
Calmodulin
DIC
C
Merge
GFP
ConA
DIC

Figure 28. Western blot analysis of TcDisgorgin/Drainin over-expressed in *T. cruzi*. 70 kDa TcDisgorgin/Drainin-GFP was detected in soluble and membrane fraction of the TcDisgorgin/Drainin over-expressing cells. A 30 kDa protein in the soluble fraction may be degraded GFP protein. S; soluble fraction, and P; membrane fraction

Figure 28.



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