THE *TOXOPLASMA GONDII* VACUOLAR PROTON ATPASE:

CHARACTERIZATION OF TWO PROTON-TRANSLOCATING SUBUNITS

by

ROXANA CINTRON-MORET

(Under the Direction of Silvia N.J. Moreno)

ABSTRACT

Toxoplasma gondii is an opportunistic organism in immunocompromised patients and an important cause of congenital disease. Our laboratory identified a new compartment in *T. gondii* which has several proteins homologous to plant vacuolar proteins, including the vacuolar proton pyrophosphatase and subunits of the vacuolar proton ATPase (V-H^+-ATPase). The V-H^+-ATPase is an enzyme involved in vacuolar acidification and vesicle trafficking in eukaryotic cells and we hypothesize it serves similar roles in the parasite. Two *a* subunits were found in the *T. gondii* genome and named as TgVHA-a1 and TgVHA-a2. We demonstrated that TgVHA-a1 localizes to the PLV, whereas TgVHA-a2 is associated with the plasma membrane of extracellular tachyzoites. Our studies revealed a possible role for the V-H^+-ATPase in the *T. gondii* secretory pathway as both *a* subunits colocalize with TgMIC2 in a *ΔTgVP1* line.

INDEX WORDS: *Toxoplasma gondii* (*T. gondii*), plant-like vacuole (PLV), V-H^+-ATPase subunit *a*, Rab 7 (TgRab7), vacuolar acidification, vesicle trafficking, endocytic pathway, bafilomycin A1, V-H^+-PPase (TgVP1), cathepsin L (TgCPL)

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DEDICATION

I dedicate this work to my beloved son Anton M. Lamorte-Cintron. Always remember to work hard and give the best and you shall see the recompense. God, thank you for the gift of knowledge and wisdom. Once again you helped me to complete a chapter in my life and start a new one with Your vision.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite of cosmopolitan distribution and the causative agent of toxoplasmosis, an opportunistic infection in immunodeficient individuals. The parasite has a unique and complex secretory system, composed of micronemes, rhoptries and dense granules, essential for host cell invasion and establishment of the parasitophorous vacuole. The mechanisms of parasite invasion have been studied extensively, however, little is known about the homeostatic mechanisms involved in parasite survival before host cell invasion. We recently identified a new compartment in *T. gondii* extracellular tachyzoites, which we named plant-like vacuole (PLV). Proteomic analysis of the PLV resulted in the identification and subsequent localization of several plant-like proteins, including a vacuolar proton pyrophosphatase (TgVP1), a cysteine protease (TgCPL) and several subunits of the vacuolar proton ATPase (V-H^+-ATPase). We hypothesize that the *T. gondii* V-H^+-ATPase helps to maintain intracellular pH homeostasis through acidification of the PLV (or acidocalcisomes) and proton extrusion in the extracellular tachyzoite stage. In order to test this hypothesis, we characterized two vacuolar proton-translocating subunits of the *T. gondii* V-H^+-ATPase by tagging them with the YFP reporter and studying their localization in *Δku80* and *ΔTgVP1* parasite lines. This research will be useful in elucidating the homeostatic mechanisms involved in *T. gondii* during acute infection and provides a model to understand the regulation of apicomplexan V-H^+-ATPases.

2. Medical importance of *Toxoplasma gondii*

Toxoplasma gondii is a cosmopolitan pathogenic protozoan that infects a wide range of vertebrates including humans. Seroprevalence of *T. gondii* in the United States is about 15%, but some European countries have higher rates[1]. Routes of parasite transmission include ingestion of tissue cysts containing highly infective bradyzoites, through contaminated food or water, contact with cat feces, congenital transmission, and in some cases by organ transplantation and blood transfusion. Acute toxoplasmosis is caused by the active tachyzoite stage that disseminates via the lymphatic and/or vascular systems and infects phagocytic and nonphagocytic nucleated cells. In immunocompetent individuals, infection is in general asymptomatic and is controlled by the immune response of the host.

T. gondii is an important cause of congenital disease, affecting fetuses from recently infected mothers, and a life-threatening opportunistic pathogen in immunocompromised patients. For example, a recent study suggested that *T. gondii* seropositivity among heart transplant recipients is associated with a significantly increased risk of cardiac mortality and higher risk of developing advanced cardiac allograft vasculopathy[2]. Toxoplasmosis in immunocompromised cardiac recipients can result from the transmission of a seropositive donor to a seronegative recipient or from the reactivation of a pre-transplant latent infection[3]. Furthermore, toxoplasmic encephalitis is the most common infection in HIV-infected patients[4]. The treatment of choice is the combination of pyrimethamine (folic acid antagonist) and sulfadiazine or clindamycin, along with folic acid. These drugs are ineffective against the chronic form of the parasite (bradyzoite). Once treatment is initiated, immunocompromised patients are required to take the drugs for life, leading to toxic side effects, like diarrhea, bone marrow suppression and liver toxicity[5]. There is no vaccine available to immunize against *T. gondii* thus it is important to deal with

toxoplasmosis by prevention or selecting available drugs taking into consideration their limitations.

3. A novel plant-like vacuolar organelle in *T. gondii*

Apicomplexan parasites have peculiar organelles which have been the subject of intense study because of their relevance to parasite biology and their potential source of novel drug targets. These unique organelles include a plastid known as the apicoplast, and a distinct apical complex consisting of different types of secretory organelles, such as the micronemes, rhoptries, and dense granules. The apicoplast is a non-photosynthetic plastid relict, which is the result of two endosymbiotic events, the first being the acquisition of a primary plastid (cyanobacterium and heterotrophic eukaryote resulting in a photosynthetic eukaryotic alga) and the second being the incorporation of the primary plastid (from photosynthetic alga) into another heterotrophic eukaryote[6-8]. We recently characterized another plant-like compartment functionally similar to plant vacuoles[9]. The *T. gondii* plant-like vacuole (PLV) is a multivesicular organelle, which contains several membrane transporters and vacuolar pumps, including the vacuolar proton pyrophosphatase (TgVP1) and the vacuolar proton ATPase (V-H^+-ATPase). Parussini et al.[10] also described the PLV (or VAC, as they named it) as well as an additional compartment known as the 'VP1 compartment'. In extracellular tachyzoites, both compartments are adjacent to each other, but can be distinguished because of the presence of a cathepsin L protease (TgCPL) in the PLV (or VAC), an hydrolase similar to other eukaryotic proteases located in acidic organelles, such as lysosomes[11].

In invading tachyzoites, the PLV compartment is structurally evident before endodyogeny (replication) but fragments after one replication event in endosome-like structures containing TgVP1. Processing and recycling of the PLV components in intracellular tachyzoites seems to

occur inside an endosome-like compartment labeled with TgRab7[9-10]. It is likely that, in extracellular tachyzoites, the 'VP1 compartment' is a pre-vacuole and the PLV is the counterpart of lytic vacuoles in plants. Physiological experiments with PLV fractions demonstrated that it can transport ions such as H^+, Ca^{2+} and Na^+. In addition, functional studies of TgVP1 overexpressing cells indicate that the PLV may provide resistance to ionic and environmental stresses in extracellular tachyzoites. Figure 1.1 shows a schematic representation of the PLV with putative pumps and transporters[9].

4. The *T. gondii* vacuolar proton pyrophosphatase (TgVP1)

Membrane-associated vacuolar proton pyrophosphatases (V-H^+-PPases) are primary proton pumps that use pyrophosphate instead of ATP as an energy source for diverse cellular transport processes[12]. In plants, V-H^+-PPases are among the most thoroughly biochemically characterized pumps[12] and consists of a single polypeptide in the conformation of a dimer (subunits of 71–80 kDa). V-H^+-PPases are present in the vacuolar membrane (tonoplast) and Golgi complex[13] of plant cells. Homologues of plant V-H^+-PPases were also discovered and characterized in trypanosomatids [14-16] and apicomplexan parasites[17-20].

There is no evidence for the existence of V-H^+-PPases in human cells or other animals, making these enzymes an ideal target for anti-parasitic drug development. Previous work from our laboratory demonstrated that TgVP1, a V-H^+-PPase in *T. gondii*, localizes to acidocalcisomes[21] and the PLV[9] of extracellular tachyzoites. Direct knockout of TgVP1 decreased the ability of extracellular tachyzoites to invade host cells (Dr. Jing Liu, personal communication).

Acidocalcisomes are acidic organelles rich in pyrophosphate and polyphosphate complexed with calcium and other cations and share similarities with lysosome-related

organelles from mammalian cells[22-23]. A TgVP1 overexpressing parasite line was more resistant to high salt concentrations (287 mM NaCl) than the wild-type RH parasites, suggesting a role of TgVP1 in ion homeostasis, in this case sodium, by sequestering it into the PLV. A similar scenario has been observed in plants, in which the activity of both V-H^+-PPase and V-H^+-ATPase is regulated by salt. The V-H^+-PPase activity increases in plants grown under high salt concentrations and the expression of several V-H^+-ATPase genes has been reported to be regulated in response to salinity stress (reviewed by Silva et al.[24]).

5. The vacuolar proton ATPase (V-H^+-ATPase)

5.1 General features of V-H^+-ATPases

Vacuolar proton ATPases are multisubunit enzymes, composed of 14 main subunits that work together in coupling ATP hydrolysis to proton translocation across the plasma membrane or membranes of intracellular organelles[25]. Domain composition of these enzymes comes from research studies on the yeast V-H^+-ATPase, characterized for the first time by Kane et al.[26] It is known that two main domains assemble or dissociate in yeast, depending on different environmental conditions, which include glucose availability[27-28] and changes in intracellular pH[29]. The integral membrane V0 domain (subunits a,c1,c2,c3,d,e) associates with the cytosolic V1 components for ATP hydrolysis (subunits A,B,D,F) and stability of the enzyme (subunits C,E,G,H)[30-31]. The structure of the yeast V-H^+-ATPase is available[25,32] with recent advances in the interaction among the smaller cytosolic subunits (E,G,H) with the subunit C[31]. Figure 1.2 shows the subunit organization and assembly of V-H^+-ATPases (figure by Toei et al.[33]).

5.2 Localization and function of subunit *a* isoforms of the V-H$^+$-ATPase

The proton-translocating subunit *a* of the V-H$^+$-ATPase has been previously characterized in several eukaryotic cells including yeast, plant, insect and mammalian cells[33-34]. Subunit *a* is one of the most important components of the V-H$^+$-ATPase complex because of its function in coupling ATP hydrolysis (N-terminal of subunit *a*) to proton translocation (C-terminal of subunit *a*)[35- 36] and targeting of the complex to specific organelles. Previous studies in yeast also showed that targeting information exists in the cytoplasmic N-terminal domain of subunit *a*[37-38]. Two *a* subunits encoded by the *Vph1* and *Stv1* genes are present in yeast and their corresponding protein products localize to different compartments. Vph1 localizes to the yeast vacuolar membrane and functions in vacuolar acidification[39], whereas Stv1 is present in the Golgi and endosomes.[29] When the *Vph1* gene is disrupted, the Stv1 subunit localizes mainly to the vacuolar membrane[29,39]. In the case of yeast *a* subunits, complete loss of V-H$^+$-ATPase activity or generation of null V-H$^+$-ATPase mutants (Vma- phenotype) is only observed when both *Vph1* and *Stv1* genes are disrupted[39- 40].

Yeast mutants lacking V-H$^+$-ATPase activity are deficient in endocytosis[29], and also sensitive to high extracellular Ca^{2+} concentrations[41] and high extracellular pH. These mutants can survive only in medium at pH 5 to 5.5 and fail to grow at pH 7 or higher[29,39,42]. Taken together, these studies show that vacuolar acidification by the yeast V-H$^+$-ATPase is essential for intracellular pH regulation[42]. Because of the functional conservation of V-H$^+$-ATPase subunits among yeast and different organisms, Vma- yeast mutants are a feasible model for the identification and characterization of these enzymes.

In mammalian cells, functions of V-H$^+$-ATPases include vesicle trafficking between the plasma membrane and organelles (exocytic pathway) and interaction with small GTPases for

molecular signaling[43]. Mammalian cells have four *a* isoforms which share 47−61% identity at the amino acid level[44]. Localization of these isoforms appear to be tissue-specific and dependent on the pH conditions required by specific cell types. For example, isoform *a3* is highly expressed in osteoclasts and localizes to the plasma membrane where it is involved in bone resorption through acid secretion mediated by the V-H^+-ATPase. However, in preosteoclasts, *a3* localizes to lysosomes, and in mature osteoclasts, *a3* localizes to the plasma membrane[45]. Mutations in the *a3* isoform have been identified in a severe form of infantile osteopetrosis, an inherited disease characterized by inadequate bone resorption[46-47].

The mechanism of bone resorption in osteoclasts depends on actin binding to the N-terminal domain of the cytosolic subunit *B2*, whereas subunit *a3* binds to the C-terminal domain of *B2*. Lack of actin binding is thought to prevent the V-H^+-ATPase from trafficking to the plasma membrane, which decreases bone resorption. This was further demonstrated by the use of the small molecule inhibitor of osteoclast resorption KM91104 (3,4-dihydroxy-N'-(2-hydroxybenzylidene)benzohydrazide) which interacts with the subunits *a3* and *B2* in an *in vitro a3-B2* binding model. The inhibitor treatment had no effect on osteoblast bone formation and no evidence of being cytotoxic in mammalian cells, making it an ideal alternative treatment of osteoporosis[48]. Isoform *a3* is also involved in vesicle acidification for proteolytic processing of insulin in vesicles of pancreatic islet cells[49]. On the other hand, the *a1* isoform plays a role on fusion between phagosomes and lysosomes during microglial phagocytosis, a mechanism of neuronal degeneration[50].

Isoform *a2* localizes to apical endosomes of the proximal tubule cells of the kidney helping in the release of endocytosed peptides from receptors, which mediates cell absorptive uptake from the renal fluid[51, 52]. The *a4* isoform is found in narrow and clear cells of the

kidney[49], the epididymis[53] and the inner ear[54]. The epididymis and vas deferens have a subpopulation of epithelial cells resembling kidney intercalated cells. These cells express very high levels of the V-H$^+$-ATPase on their apical membrane and intracellular vesicles[55] to keep a low luminal pH and low bicarbonate concentration that are critical for spermatozoa maturation and storage in the epididymis[44,56]. Mutations in V-H$^+$-ATPase subunit isoforms *B1* and *a4* were found in patients with inherited forms of distal renal tubular acidosis with and without sensorineural deafness[44,57]. Distal renal tubular acidosis results from a direct failure of the distal nephron to secrete acid into the tubular lumen resulting in the inability to acidify the urine below pH 5.5 in the setting of metabolic acidosis (reviewed by Wagner[44]).

5.3 Regulation of the V-H$^+$-ATPase activity

The luminal pH of compartments along the endocytic pathway decreases while following the path from early endosomes to lysosomes. Endocytic processess include uncoupling of ligands from their receptors in early endosomes, budding of endocytic carrier vesicles that move ligands from early to late endosomes, and degradation of endocytosed macromolecules in lysosomes (reviewed by Kumari[58]). Organellar acidification and vesicle trafficking through the endocytic pathway is a well described function of the V-H$^+$-ATPase[33]. Regulation of the V-H$^+$-ATPase depends on the luminal pH of the organelle where the proton-translocating subunit *a* is residing, and the intracellular and extracellular pH[27,59-62]. Two common mechanisms of regulation are (1) dissociation and reassembly of the membrane V0 and cytoplasmic V1 sectors and (2) change in pump density through reversible fusion of V-H$^+$-ATPase-containing vesicles, most often with the plasma membrane (reviewed by Toei et al.[33]).

Reversible dissociation occurs rapidly and does not require new protein synthesis[27-28, 38]. Upon dissociation, the V1 cytosolic sector is uncoupled in ATP hydrolysis by

interaction with subunit *H* and released into the cytosol[63-64]. This results in an unregulated passive proton leak in cellular membranes effected by the V0 sector[65]. In yeast, V-H$^+$-ATPase complexes localized to the vacuolar membrane dissociate upon glucose depletion[27], whereas Stv1-containing complexes localized to the Golgi do not dissociate unless these complexes localize to the vacuole by overexpression of the *Stv1* gene[36] Yeast cells starved for glucose have an intracellular pH of 6.0 that is more acidic than the pH in cells fermenting glucose (pH of 7.2)[66].

Dissociation requires an intact microtubular network[67] whereas reassembly, but not dissociation, requires the protein complex RAVE[68], which appears to stabilize the dissociated V1 complex in an assembly competent form[69]. In yeast, binding of the glycolytic enzyme aldolase with V-H$^+$-ATPase subunits *a*, *B*, and *E* was previously demonstrated[70-71]. Glucose causes assembly of the V-H$^+$-ATPase by activating the Ras/cAMP/protein kinase A pathway[28,72]. Elevated glucose levels increase GTP-bound Ras, which activates cAMP production by adenylate cyclase. Elevated cAMP levels cause dissociation of the regulatory subunits of protein kinase A promoting phosphorylation of a serine residue in the cytoplasmic A subunit, the ATP hydrolysis component of the V1 domain[61, 72-73]. Thus, the Ras/cAMP/PKA pathway is suppressed upon glucose depletion, leading to V-H$^+$-ATPase dissociation. In insect cells, protein kinase A stimulates assembly of the V-H$^+$-ATPase by phosphorylation of subunit C[74]. In renal epithelial cells, phosphatidylinositol 3-kinase is one of the signalling molecules required for V-H$^+$-ATPase assembly and trafficking[75].

5.4 V-H$^+$-ATPase localization and function in pathogenic Protozoa

The identification of a V-H$^+$-ATPase in *T. gondii,* its localization and role in intracellular pH regulation in extracellular tachyzoites, was first addressed by Moreno et al.[76]. The *T. gondii* V-H$^+$-ATPase was localized to the plasma membrane and intracellular vacuoles by

labeling of extracellular tachyzoites with an antibody against several subunits from the V-H$^+$-ATPase of *Dictyostelium discoideum*[9,76]. Similar localization results were obtained for the V-H$^+$-ATPase in all stages of *Trypanosoma cruzi*[14,77]. Studies in *Plasmodium falciparum* trophozoites revealed that the V-H$^+$-ATPase localizes in membranous structures and the plasma membrane of the erythrocyte and functions in balancing the intracellular pH of the host[78]. Hayashi et al.[79] also demonstrated that the *P. falciparum* V-H$^+$-ATPase actively extrudes protons from the parasite's plasma membrane to a region in the host cytoplasm.

Previous work in our laboratory demonstrated that PLV-enriched fractions have ATP-driven transport, which is inhibited by bafilomycin A1[9], a specific inhibitor of V-H$^+$-ATPases when used at nanomolar concentrations[80-81]. The V-H$^+$-ATPase inhibitors bafilomycin A1 and concanamycin A are macrolide antibiotics known to specifically interact with two V0 domain components, the proteolipid ring (*c* subunits) and subunit *a* of the enzyme, specifically inhibiting catalysis and rotation of the c subunit ring. The consequence of the binding of the V-H$^+$-ATPase inhibitor to the V0 components *c* and *a* is alkalinization of the organelle where the enzyme complex resides by disruption of its functional role in organelle acidification[82-84]. Parussini et al.[10] reported that extracellular tachyzoites exhibited a size increase of the PLV upon addition of bafilomycin A1. It is possible that addition of bafilomycin A1 to extracellular tachyzoites promotes alkalinization of the PLV leading to accumulation of proteins and other cellular components in the PLV lumen[11, 85].

Evidence of organellar alkalinization in the presence of bafilomycin A1 has been reported in acidocalcisomes of trypanosomes and apicomplexan parasites. Acidocalcisomes in these parasites contain a V-H$^+$-PPase and a V-H$^+$-ATPase[86-90]. In addition, *T. gondii* tachyzoites contain high levels of long and short chain polyphosphate (PolyP), as determined biochemically

with a protocol that involves the activity of a recombinant yeast exopolyphosphatase. The concentration of polyP changes drastically under alkaline stress or when the cells are incubated with Ca^{2+} ionophores. For example, the addition of bafilomycin A1 leads to acidification of the cytosol and alkalinization of the acidocalcisomes by inhibition of the V-H^+-ATPase. Addition of bafilomycin A1 or the alkalinizing agent NH_4Cl to tachyzoites also resulted in significant decrease in long and short chain polyP[87]. Influx of ammonia into tachyzoites induced a rapid alkalinization of the cells followed by recovery of the cytoplasmic pH, which occurred in parallel with PolyP hydrolysis[76,87]. It is known that proton generation from polyP hydrolysis can neutralize up to 2.5 pH units of change in the yeast *Saccharomyces cerevisiae*[91] suggesting a role of polyP hydrolysis in cytosolic pH recovery. In *T. gondii* cells incubated with ionomycin (and EGTA to avoid Ca^{2+} entry), Ca^{2+} release from acidocalcisomes was also associated with the hydrolysis of polyP[87].

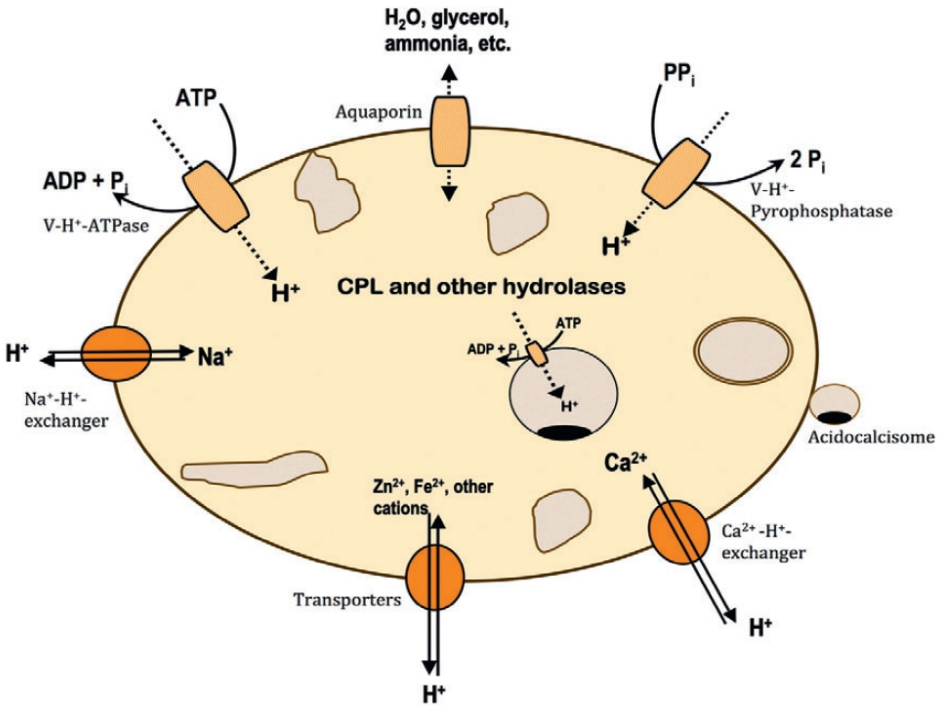


Figure 1.1 Schematic representation of the plant-like vacuole. The H^+ gradient is established by a vacuolar proton ATPase (V-H^+-ATPase) and a vacuolar proton pyrophosphatase (V-H^+-PPase). An aquaporin channel would transport water or other osmolytes that could help the parasite deal with environmental stress. Other potential transporters include Na^+/H^+ and Ca^{2+}/H^+ exchangers. As in the plant vacuole, other transporters may be present, which could transport other cations using the proton gradient generated by the proton pumps. Some internal vesicles and acidocalcisomes are also shown (figure from Miranda et al.[9]).

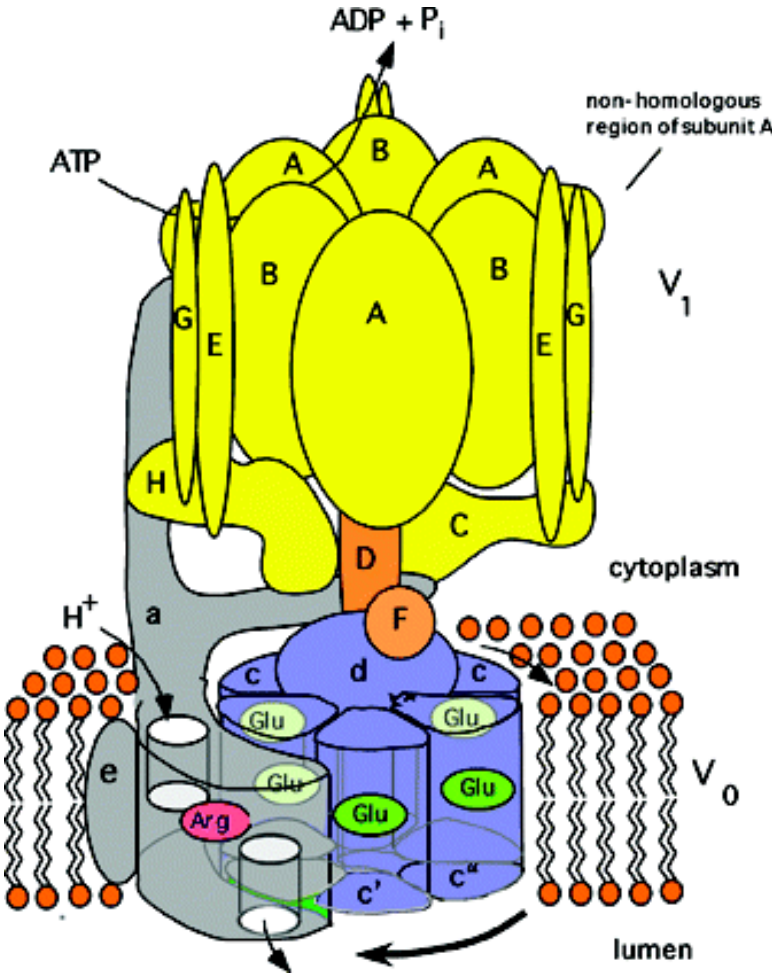

ADP + Pi
non-homologous region of subunit A
ATP
A
B
A
B
B
A
G
E
E
G
V1
H
C
D
cytoplasm
H+
a
F
c
d
c
Glu
Glu
e
Glu
Glu
Glu
Arg
c'
c"
V0
lumen

Figure 1.2 Structure and mechanism of the V-H^+-ATPase. The V-H^+-ATPase is composed of two domains, V1 and V0. The peripheral V1 domain is composed of eight different subunits (*A-H*, *yellow* and *orange*) and is responsible for ATP hydrolysis, whereas the integral V0 domain is composed of six subunits (in yeast, subunits *a, c, c', c", d,* and *e*, *blue* and *gray*) and is involved in the translocation of protons across the membrane. ATP hydrolysis drives the rotation of a central rotor, which is composed of the *D*, *F, d*, and proteolipid (*c, c'* and *c"*) subunits. Subunit *a* possesses two hemichannels and a crucial arginine residue (*red*), which are required for proton translocation. The hemichannels allow protons to reach buried glutamic acid residues (*green*) on the proteolipid ring from the cytoplasmic side of the membrane and to leave from these sites to the luminal side of the membrane following interaction of the glutamate residues with the *a* subunit arginine residue. The V1 and V0 domains are connected by a central stalk, which is composed of subunits *D, F*, and *d*, and three peripheral stalks, which are composed of subunits *C, E, G, H* and the N-terminal cytoplasmic domain of subunit *a*. The peripheral stalks hold the A3B3 hexamer stationary with respect to subunit *a*. The nonhomologous region of subunit *A*, which is absent from the F1F0 ATP synthases, is involved in reversible dissociation (text and figure from Toei et al.[33]).

CHAPTER 2

LOCALIZATION OF TWO V-H$^+$-ATPASE PROTON-TRANSLOCATING SUBUNITS IN THE TACHYZOITE STAGE OF *TOXOPLASMA GONDII*

2.1 Objective

We previously confirmed the presence and transport activity of a V-H$^+$-ATPase in *T. gondii*[9]. Moreover, a polyclonal antibody raised against several V-H$^+$-ATPase subunits from *Dictyostelium discoideum* localized to the PLV and the plasma membrane of extracellular tachyzoites. We found two proton-translocating subunits *a* in the *T. gondii* genome and sought to establish their localization in order to predict their function in the parasite. Peptides belonging to subunit TgVHA-a1 were found in PLV-enriched fractions. Based on the analysis of the PLV proteomic data, our hypothesis is that the TgVHA-a1 subunit localizes to the PLV whereas TgVHA-a2 is present in the plasma membrane. In order to test this hypothesis, endogenous gene tagging of both *a* subunits was performed as the initial step to localize these proteins in the tachyzoite stage.

2.2 Materials and Methods

2.2.1 Sequence analysis

The *T. gondii* TgVHA-a1 (TGME49_032830) and TgVHA-a2 (TGME49_090720) protein sequences were retrieved from ToxoDB (version 6.4). The yeast VPH1 (CAA99494.1) and STV1 (CAA89764.1) amino acid sequences were downloaded from GenBank. A multiple sequence alignment was created using ClustalW (http://www.genome.jp/tools/clustalw/) and visualized in GeneDOC (http://www.psc.edu/biomed/genedoc).

2.2.2 Parasite Lines and Maintenance

Toxoplasma gondii Δ*ku80* and Δ*TgVP1* tachyzoites were grown in h-Tert human fibroblasts[92] as described before[93]. The Δ*ku80* line lacks the *Ku80* gene involved in nonhomologous end-joining (NHEJ) DNA repair, increasing homologous recombination into the correct locus. These cells were grown in DMEM media containing 1% FBS plus the required drug selection for maintenance of resistant parasite lines. TgVHA-a1-YFP and TgVHA-a2-YFP tachyzoites were selected with 1 μM pyrimethamine (Sigma). Selection of mutant parasite lines for the knockout and overexpression studies was done using 20 μM chloramphenicol (Sigma). For semisynchronized parasite cultures, h-Tert cells cultured in 75 cm^2 flasks, were infected with 3.7 x 10^7 tachyzoites/flask for 2 h, extracellular parasites thoroughly washed and the cultures allowed to grow for 35–40 h. At this time, extracellular parasites were removed by washing with fresh IM (DMEM containing 20 mM HEPES, pH 7.4 with 1% FBS) three times and the cultures allowed to grow for two additional hours in IM to keep the pH close to 7.2. Subsequently, the extracellular tachyzoites were washed off and the intracellular tachyzoites collected in fresh IM by scrapping off the host monolayer and purifying the parasites by filtration through a nucleopore membrane. The released intracellular tachyzoites were resuspended in buffer A with glucose (BAG) (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 50 mM HEPES, pH 7.2, 5.5 mM glucose).

2.2.3 Generation of *TgVHA-a1-YFP* and *TgVHA-a2-YFP* transgenic parasites

The pYFP-LIC-DHFR vector was used for endogenous gene tagging, which was kindly provided by Dr. Vern Carruthers. Primers were designed to contain the LIC sequence and used to amplify the 3'-end of *TgVHA-a1* and *TgVHA-a2* by PCR. The genes were inserted in frame with the *YFP* gene in the pYFP-LIC-DHFR vector by ligation- independent cloning (LIC).

Parasites were collected, centrifuged and resuspended in cytomix buffer (2 mM EDTA, 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM HEPES, 5 mM $MgCl_2 \cdot 6H_2O$; pH 7.6). Fifteen micrograms of purified recombinant vector were electroporated into Δ*ku80* parasites using a BioRad GenePulser X-Cell electroporator (settings of 1.5 kV, 25 µF, 25 Ω). Positive selection with pyrimethamine (*DHFR-TS* marker) was done as previously described by Huynh and Carruthers[94]. Subcloning was done in 96-well plates by fluorescence activated cell sorting (FACS) at the CTEGD flow cytometry core facility to isolate YFP positive parasites.

2.2.4 Western blot analysis

Extracellular tachyzoites were collected and lysed directly in non-boiled Laemli sample buffer plus 20% 2-mercaptoethanol. The samples (5 x 10^7 parasites/lane) were loaded into 8% sodium dodecyl sulfate-polyacrylamide gels for electrophoresis and transferred to a nitrocellulose membrane for 2 h. Blocking was done in 5% non-fat dry milk in PBS-T (1X PBS, pH 7.2 and 0.1% Tween-20) overnight. The membranes were probed with rabbit anti-GFP (1:10000) antibody from Invitrogen for 1 h, washed three times for 15 min, and probed 1 h with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Biorad).

2.2.5 Immunofluorescence Microscopy

Tachyzoites were harvested and washed with BAG buffer and fixed with 4% formaldehyde for 1 h. Immunofluorescence assays were performed as described[21] by using primary antibodies at the concentrations indicated in the legends. Secondary antibodies used were Alexa 488 and Alexa 568 conjugated anti-rabbit IgG or Alexa 488 and Alexa 546 conjugated anti-mouse (Molecular Probes). Fluorescence images were collected with an Olympus IX-71 inverted fluorescence microscope with a Photometrix CoolSnapHQ CCD camera driven by DeltaVision software (Applied Precision, Seattle, WA). Collected images were

deconvolved using Softworx deconvolution software (Applied Precision, Seattle, WA). For all images, 15 cycles of enhanced ratio deconvolution were used.

2.2.6 Measurements of proton extrusion

TgVHA-a1-YFP or Δ*ku80* extracellular tachyzoites were collected and resuspended to a final density of l0^{9} cells ml^{-1} in BAG, pH 7.3 and kept on ice. To initiate measurements, BCECF (0.38 µM final concentration) and 100 µl parasite suspension were added to 2.45 ml of a low buffering solution (135mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1mM $MgSO_4$, 5mM glucose) to detect extracellular pH changes. Slope values were calculated as the change in extracellular pH (pHe) per second.

2.3 Results

2.3.1 The *T. gondii* V-H^{+}-ATPase *a* subunits contain proton-translocating conserved amino acid residues

A multiple sequence alignment was performed to compare the *T. gondii* V-H^{+}-ATPase with the yeast *a* subunits (Fig 2.1). The alignment showed that 11 from the thirteen aminoacids important for proton-translocation, including the essential arginine (Fig 1.2, *red circle*), which interacts with the glutamic acid residues (Fig 1.2, *green circles*) embedded in the proteolipid ring of the enzyme, are conserved in the *T. gondii a* subunits (Fig 2.1, *yellow* and *blue* boxes). The sequences share an average of 27% identity with yeast and show highest conservation in the transmembrane domain regions (TMD). The essential arginine is found in TMD7 when compared to the yeast subunit *a* sequences (Fig 2.1). Appendix A includes Table A1 containing the V-H^{+}-ATPase subunits found in *T. gondii*.

2.3.2 TgVHa-a1-YFP localizes to the PLV and vesicles in extracellular tachyzoites

The V-H^+-ATPase regulation is, in part, dependent on the concerted interaction of the subunits from the V0 and V1 domains. In order to study the localization of the two *a* subunits found in *T. gondii* without altering their endogenous expression, we decided to tag the endogenous *TgVHA-a1* and *TgVHA-a2* subunit genes by ligation-independent cloning (described under Methods). Specific primers were generated to amplify the 3'-end of the gene and contained the LIC sequence for insertion into the pYFP-LIC-DHFR vector. This vector was used to transform Δ*ku80* parasites in order to tag the 3'-end of each endogenous gene with the *YFP* reporter gene. TgVHA-a1-YFP parasites were sorted by FACS and tested for integration of the cloned fragment by PCR. The size of the *TgVHA-a1* gene is 5 kb. The expected band shift (~5.2 kb because reverse primer covers first 200 bp of *YFP*) is shown confirming that the cloned *TgVHA-a1* fragment containing the *YFP* gene was inserted into the *T. gondii* genome *(*Fig 2.2). Western blot analysis of total parasite lysates show a band of approximately 130 kDa, corresponding to the size of TgVHA-a1 (101 kDa) and YFP (27 kDa) (Fig 2.2). TgVHA-a2-YFP parasites were sorted by FACS and integration of the cloned fragment was confirmed by PCR. The size of the *TgVHA-a2* gene is 10 kb, thus only a primer covering upstream the LIC site (~700 bp) was used to test for integration by PCR. The expected band shift is shown (~1.1 kb because reverse primer covers the entire *YFP* gene) (Fig 2.3).

An anti-GFP antibody was used for localization studies because the YFP signal was not strong enough to be detected by direct fluorescence. TgVHA-a1-YFP localized to vesicles and the PLV. Localization to the plasma membrane of the parasite was more evident in recently released tachyzoites (Fig 2.4, *left panel*) than in extracellular parasites incubated in BAG buffer for 30 minutes. In these cells, TgVHA-a1-YFP localized mainly to the PLV (Fig 2.4,

right panel). Labeling of the PLV and the surface of invading tachyzoites was observed before replication. After one replication event, localization to the plasma membrane of intracellular tachyzoites was mainly observed (Fig 2.5). To demonstrate that the localization of TgVP1 was not altered in TgVHA-a1-YFP intracellular tachyzoites, labeling with the anti-TgVP1 antibody was done. The characteristic PLV fragmentation previously shown in wild-type RH intracellular parasites[9] was also observed in TgVHA-a1-YFP parasites labeled with the anti-TgVP1 antibody demonstrating that PLV fragmentation after tachyzoite replication was not affected.

To show the localization of TgVHA-a1-YFP in recently egressed tachyzoites, we performed a semisynchronization protocol in which extracellular tachyzoites were washed off and the intracellular tachyzoites were released from the host cells and collected in fresh IM media (Fig 2.6A). We considered the released intracellular tachyzoites as recently egressed because they are collected immediately while in the intracellular stage. Our previous work in the characterization of the PLV demonstrated that the TgVP1 proton pump localized to the PLV in recently egressed tachyzoites[9]. However, TgVHA-a1-YFP localized in vesicles at the plasma membrane in recently egressed tachyzoites, as confirmed by comparing its localization with the plasma membrane marker TgSAG1 (Fig 2.6B). These vesicles localize to the PLV after the parasite is in the extracellular milieu for a longer period of time, as shown in Fig 2.4. These results demonstrate that TgVHA-a1-YFP changes its localization from the plasma membrane to the PLV during the transition from the intracellular to extracellular tachyzoite stage. In addition, TgVHA-a1-YFP localizes to the PLV and the surface of intracellular parasites before replication indicating a regulated translocation of TgVHA-a1 to the plasma membrane. Taken together, the data suggests that TgVHA-a1 has a functional role in the PLV, probably vacuolar acidification, as it mainly localizes there in extracellular tachyzoites.

2.3.3 TgVHA-a1-YFP is physiologically similar to the parental Δ*ku80*

Since gene tagging with YFP could affect the function of the gene product, a way to determine physiological differences between the lines studied is by spectrofluorimetric measurements of proton extrusion using a fluorescent probe. Changes in extracellular pH were detected using the fluorescent BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) probe (Invitrogen). The fluorescence ratios of BCECF, with excitation at 505 and 404 nm and emission at 530 nm, were recorded and converted into pH values on the basis of the ratios obtained from a standard pH curve with pH values ranging from 5.5 to 8.0. Figure 2.7 are representative of two independent proton extrusion measurements done in Δ*ku80* (Fig 2.7A) or TgVHA-a1-YFP (Fig 2.7B) parasites pre-incubated with the V-H+-ATPase inhibitor bafilomycin A1 or without the drug (i.e. DMSO).

Proton extrusion rates were similar between Δ*ku80* (average rate -7.7 x 10^{-4} pHe units s^{-1}) and TgVHA-a1-YFP (average rate -7.5 x 10^{-4} pHe units s^{-1}) extracellular tachyzoites. In parasites pre-incubated with 500 nM bafilomycin A1, the average rates of proton extrusion decreased significantly in both parasite lines (average rates were -5.1 x 10^{-4} and -4.5 x 10^{-4} pHe units s^{-1} for Δ*ku80* and TgVHA-a1-YFP parasites, respectively). TgVHA-a1-YFP exhibited similar sensitivity to bafilomycin A1 (~40% decrease) compared to Δ*ku80* (~34% decrease). These data suggest that tagging of *TgVHA-a1* with *YFP* did not affect the proton pumping function of the protein, although intracellular pH measurements are needed to verify this further.

2.3.4 TgVHA-a2-YFP localizes to vesicles at the plasma membrane in extracellular tachyzoites

We were interested in investigating the localization of the TgVHA-a2 subunit because according to our hypothesis it might localize to the plasma membrane of extracellular

tachyzoites. With this aim, we first created a TgVHA-a2-YFP cell line (described under Methods) and performed IFA analysis using anti-GFP antibodies. Indeed, TgVHA-a2-YFP localized to vesicles at the plasma membrane, as shown by comparing its localization with antibodies against the plasma membrane marker TgSAG1 (Fig 2.8A) and the surface adhesin (microneme protein) TgMIC2. In intracellular tachyzoites, TgVHA-a2-YFP localized to the plasma membrane and an intracellular compartment, which was also labeled with anti-TgMIC2 antibody (Fig 2.8B). No clear localization of TgVHA-a2-YFP was observed in intracellular tachyzoites after replication although the localization of TgMIC2 was not affected (data not shown). To determine if the localization of TgVHA-a2 changes during the transition from intracellular to extracellular tachyzoites as is the case of TgVHA-a1-YFP, a semisynchronization protocol was done to obtain recently egressed tachyzoites (see Materials and Methods). TgVHA-a2-YFP also displayed a punctate pattern in recently egressed tachyzoites (Fig 2.8C).

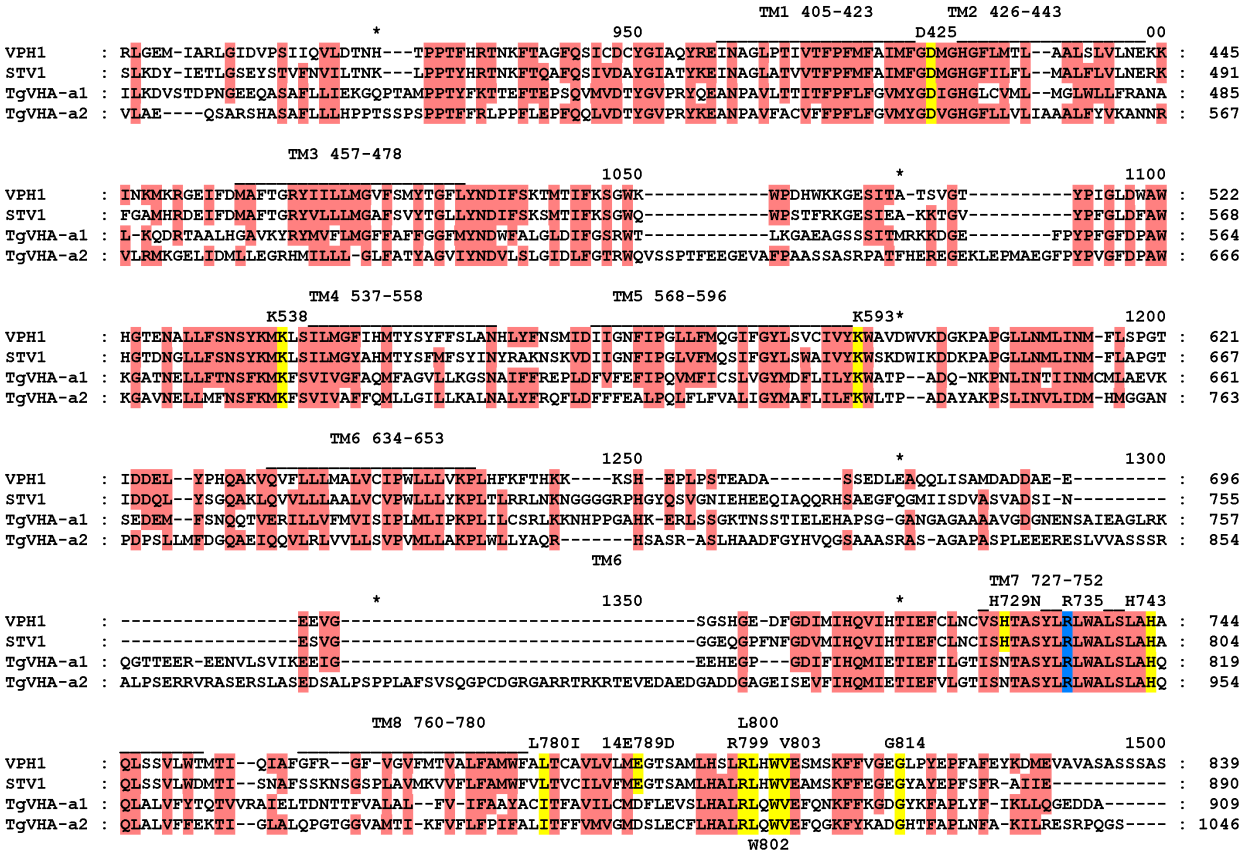
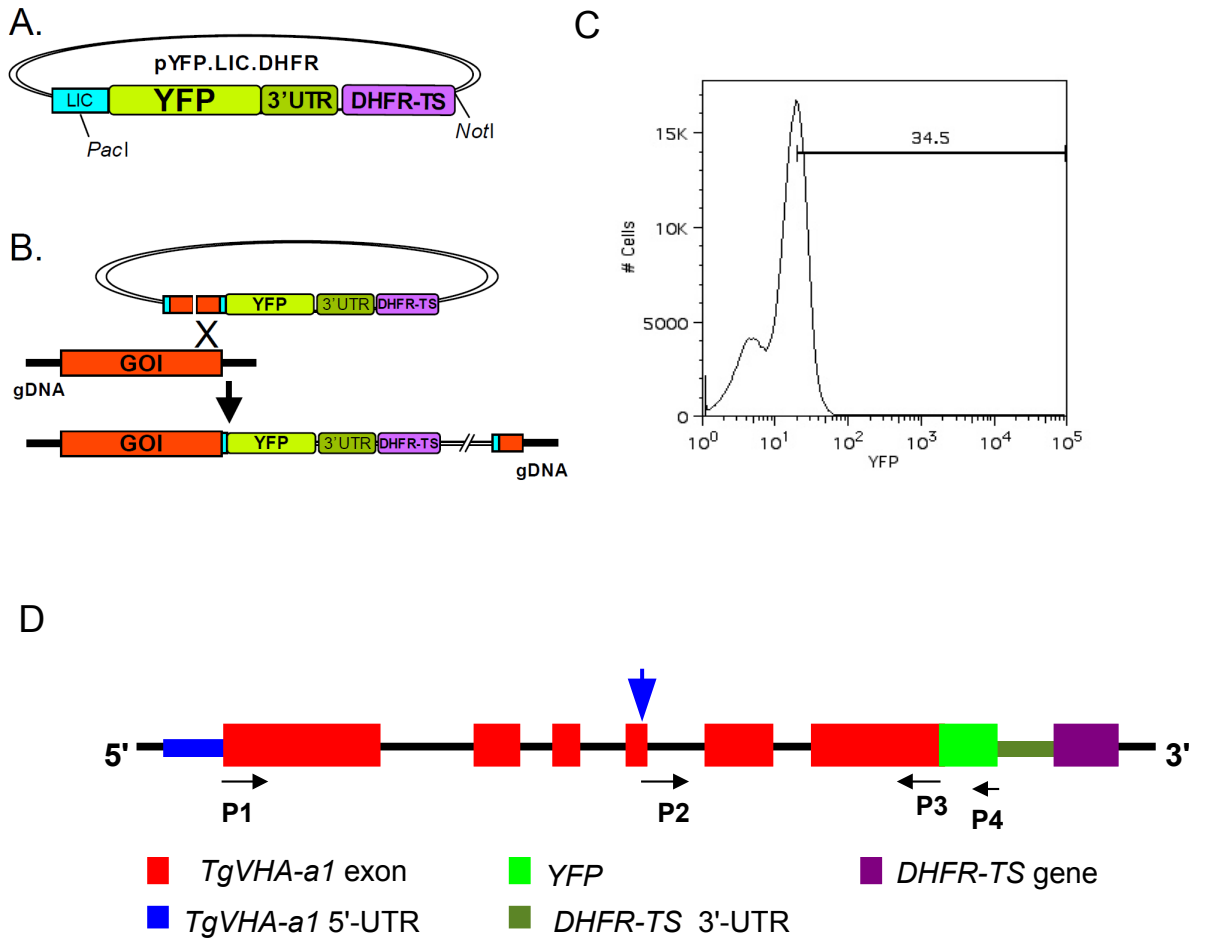


Figure 2.1 Sequence alignment comparison of the transmembrane domain region (TMD) between yeast and *T. gondii* V-H$^+$-ATPase *a* subunits. Eleven amino acids described to be important for proton-translocation in yeast are conserved in both *T. gondii a* subunits (*yellow* boxes). An amino acid change (H729N) is present in *T. gondii*. The arginine residue essential for the interaction with the glutamic acid of the proteolipid ring is conserved in *T. gondii* (*blue* box). The average percent of identity between yeast and the *T. gondii* sequences is 27%. Amino acids with >70% identity are shown in *red* boxes. Eight TMDs are represented with lines above the alignment.


A.
pYFP.LIC.DHFR
LIC
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DHFR-TS
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NotI
B.
YFP
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gDNA
GOI
YFP
3'UTR
DHFR-TS
gDNA
C
34.5
15K
10K
5000
0
Cells
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10^1
10^2
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10^4
10^5
YFP
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P4
TgVHA-a1 exon
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DHFR-TS gene
TgVHA-a1 5'-UTR
DHFR-TS 3'-UTR
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120
100
80
60
50
40
30
20
TgVHA-a1-YFP
GFP

Figure 2.2 Endogenous gene tagging and expression of *TgVHA-a1-YFP*. (A) pYFP-LIC-DHFR vector containing an upstream LIC cassette for cloning genes fused to YFP. (B) Single-crossover mechanism of integration of *YFP* to the 3'-end of a gene of interest. (A) and (B) from Huynh and Carruthers[94]. (C) FACS sorting of TgVHA-a1-YFP parasites (35% of YFP positive population was sorted). (D) Graphic illustration of *TgVHA-a1* (red) fused with *YFP* (green). *Blue arrow* points to the site used for integration. *Black arrows* indicate the primers used for the PCR to confirm integration (E). Lane M, 1 kb DNA ladder (New England Biolabs); lane 1, primers 1,3 (~5 kb); lane 2, primers 1,4 (~5.2 kb because reverse primer covers first 200 bp of *YFP*); lane 3, negative control including pYFP-LIC-DHFR vector primer and primer 4; lane 4, primers 2,3 (~2.1 kb). (F) Western blot of TgVHA-a1-YFP tachyzoite lysates. Commercial primary rabbit anti-GFP (1:10000) and HRP-conjugated secondary (1:15000) antibodies were used to detect the bands with expected size ~128 kDa (lane 1), corresponding to predicted size of 101 kDa for TgVHA-a1 and 27 kDa for YFP. A *T. cruzi* lysate expressing GFP was used as positive control (lane 3). MagicMark Protein Standard was loaded in lane 2.

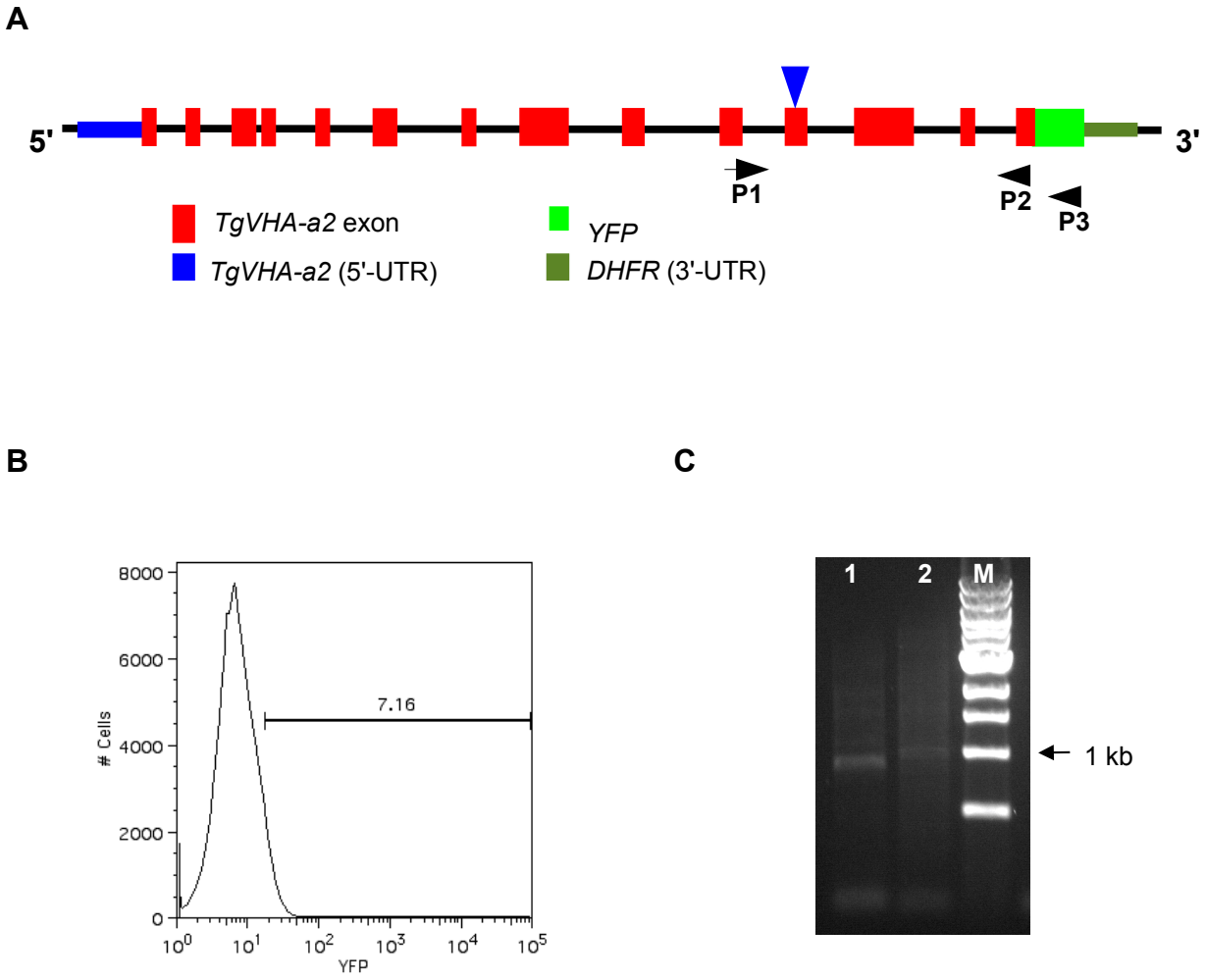


Figure 2.3 Endogenous gene tagging of *TgVHA-a2-YFP*. (A) Graphic illustration of *TgVHA-a2* (red) fused with *YFP* (green). The predicted size of the gene is ~10 kb. *Blue arrow* points to the site of integration. Black arrows indicate primers used to test for integration by PCR. (B) FACS sorting of TgVHA-a2-YFP parasites (YFP positive population represented ~7% of the total parasite population). (C) PCR to confirm integration of the cloned fragment in the *TgVHA-a2* locus. Lane M, 1 kb DNA ladder (New England Biolabs); lane 1, primers 1,2 (~0.7 kb); lane 2, primers 1,3 (~1.1 kb).

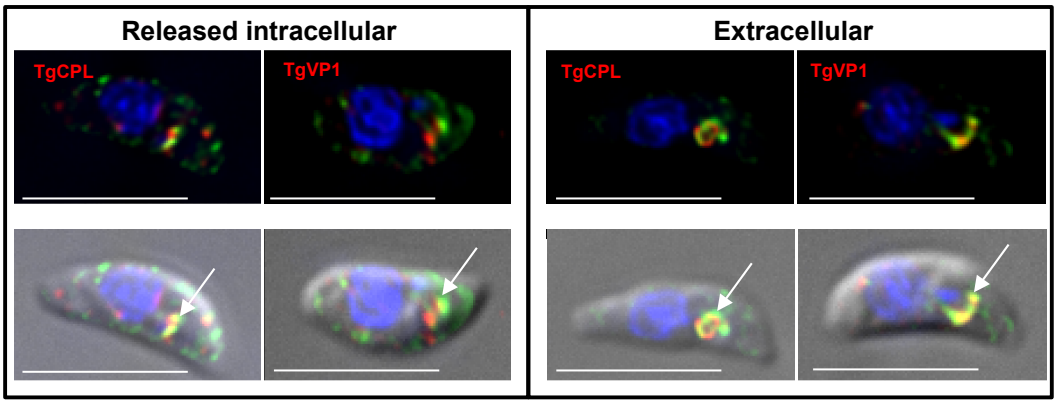


Figure 2.4 Localization of TgVHA-a1-YFP in extracellular tachyzoites. Parasites were collected at 60/40 extracellular/intracellular tachyzoite ratio. Extracellular parasites were harvested in BAG for 30 min before fixing *(right panel)*. Intracellular tachyzoites were mechanically released by syringe, centrifuged and fixed *(left panel)*. Labeling with anti-GFP antibody (1:2000, green) and anti-TgVP1 (1:4000, red) or anti-TgCPL (1:400, red) antibody is shown. DAPI staining of the nucleus is shown (blue). *Arrows* point to the PLV compartment. Scale bars = 5 μm.

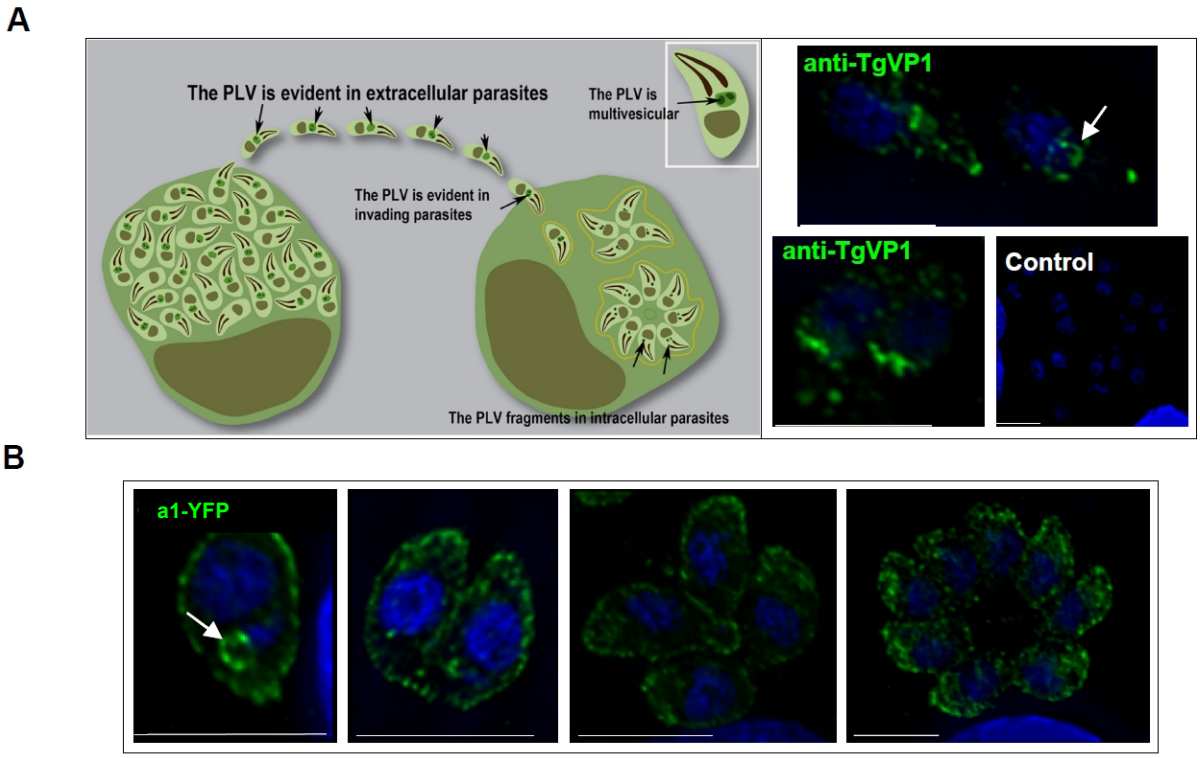
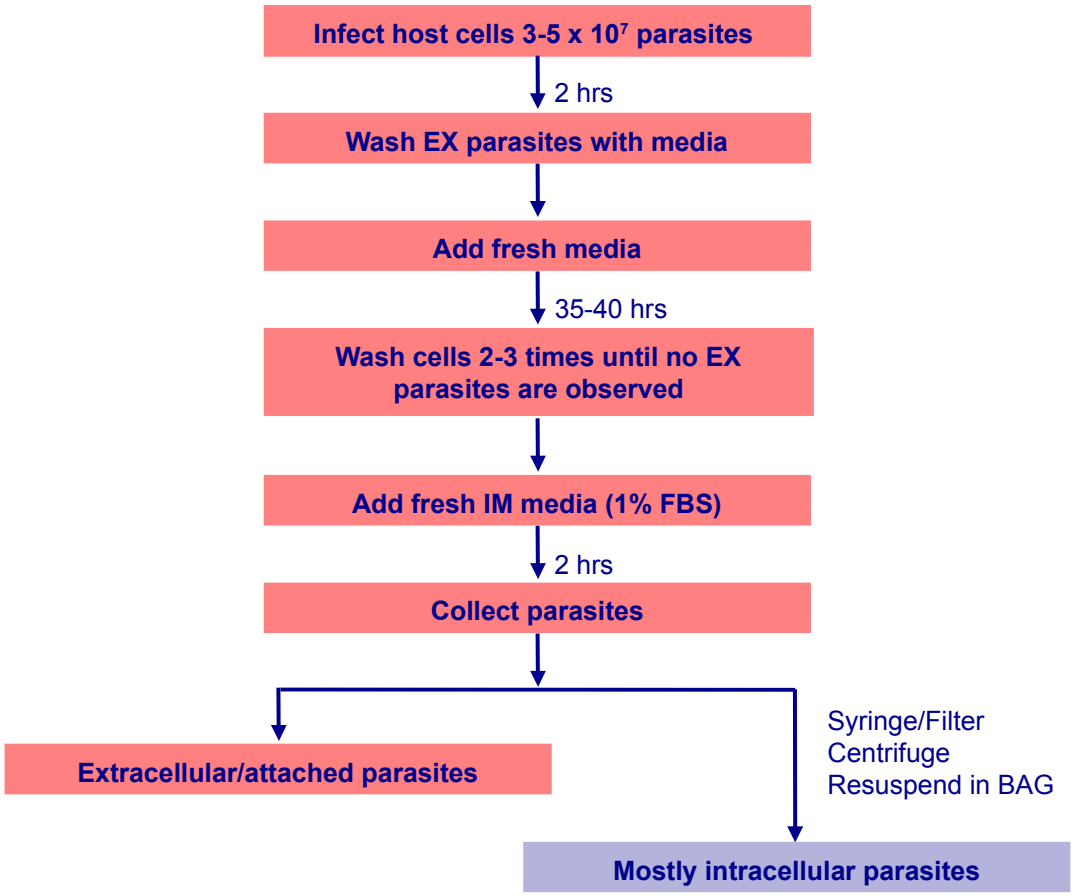


Figure 2.5 Localization of TgVHA-a1-YFP in intracellular tachyzoites. Human h-Tert cells were grown on coverslips in 12-well plates and infected with 10^7 TgVHA-a1-YFP tachyzoites overnight. Immunofluorescence staining was performed as described in Materials and Methods. (A, *left panel*) Model for the PLV appearance during the lytic cycle of *T. gondii.* The PLV structure is maintained immediately after invasion, and fragments during intracellular replication[9] (A, *right panel*). Labeling with anti-TgVP1 antibody. No YFP signal was observed in fixed tachyzoites stained with secondary antibody only (control). (B) Staining with anti-GFP antibody. *Arrows* point to the PLV. DAPI staining of the nucleus is shown (blue). Scale bars = 5 µm.

A



B

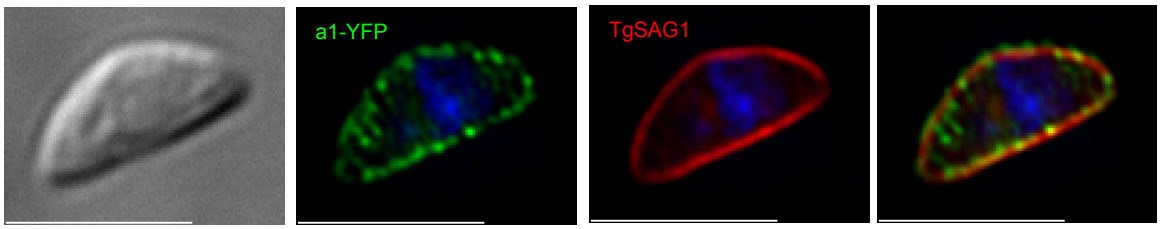


Figure 2.6 Localization of TgVHA-a1-YFP in recently egressed tachyzoites. (A) Semi-synchronization protocol to obtain recently egressed tachyzoites. (B) Indirect immunofluorescence of recently egressed tachyzoites. Labeling was performed with anti-GFP (1:2000, green) and plasma membrane marker anti-TgSAG1 (1:2000, red) antibodies. DAPI staining of the nucleus is shown (blue). Scale bars = 5 µm.

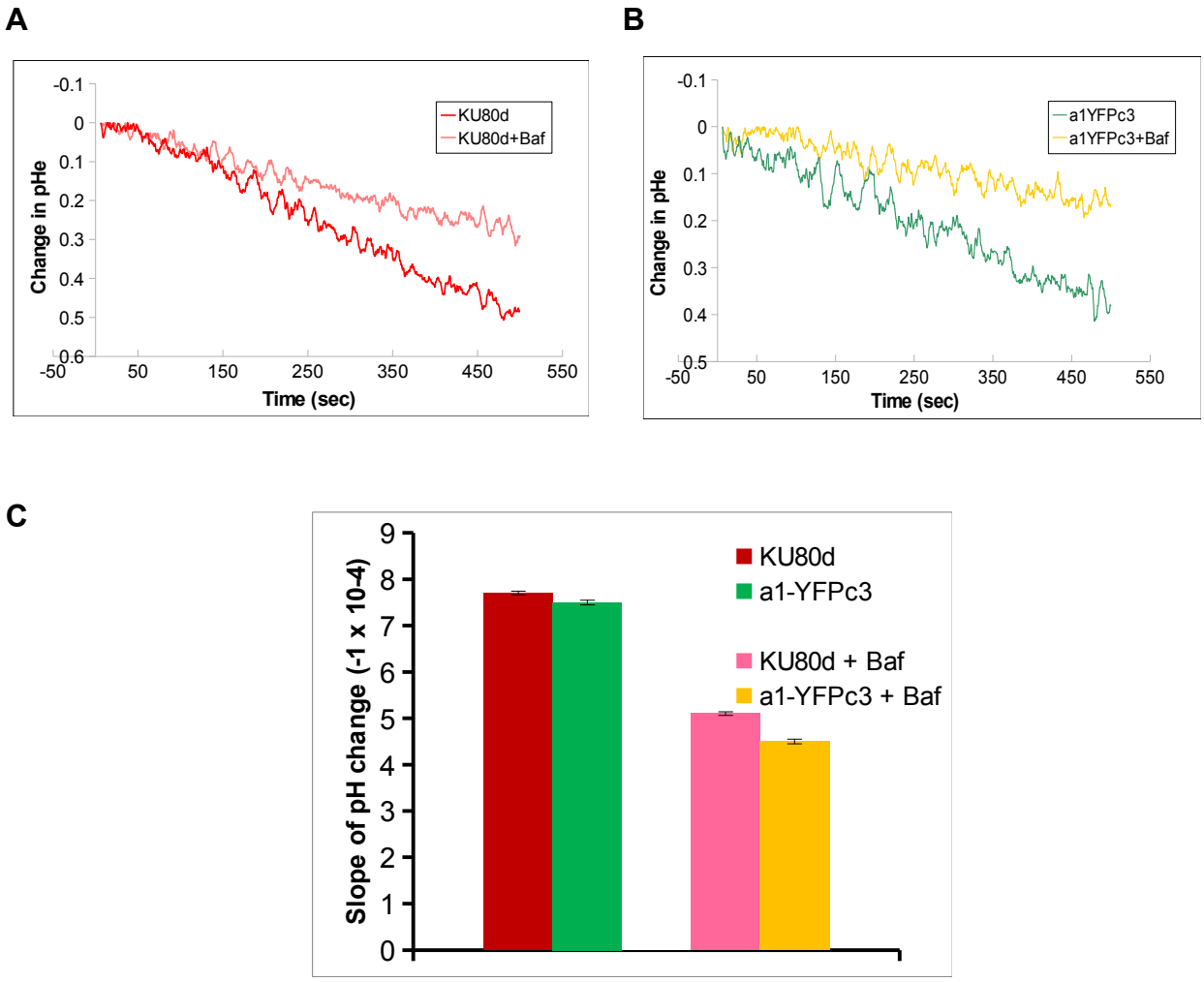


Figure 2.7 Proton extrusion in Δ*ku80* and TgVHA-a1-YFP extracellular tachyzoites. Δ*ku80* or TgVHA-a1-YFP extracellular tachyzoites were resuspended to a final density of l0^{9} cells ml^{-1} in BAG, pH 7.3 and kept on ice. BCECF was added to a parasite suspension in a low buffering solution to detect extracellular pH changes. (A,B) Representative tracings of changes in extracellular pH (pHe) in Δ*ku80* (A) or TgVHA-a1-YFP (B) tachyzoites after pre-incubation or no treatment with 500 nM bafilomycin A1. (C) Slope values (change in pHe per second) in tachyzoites pre-incubated or not treated with the inhibitor. Measurements are the result of 3 separate tracings done the same day. The error bars represent the standard error of the slope.

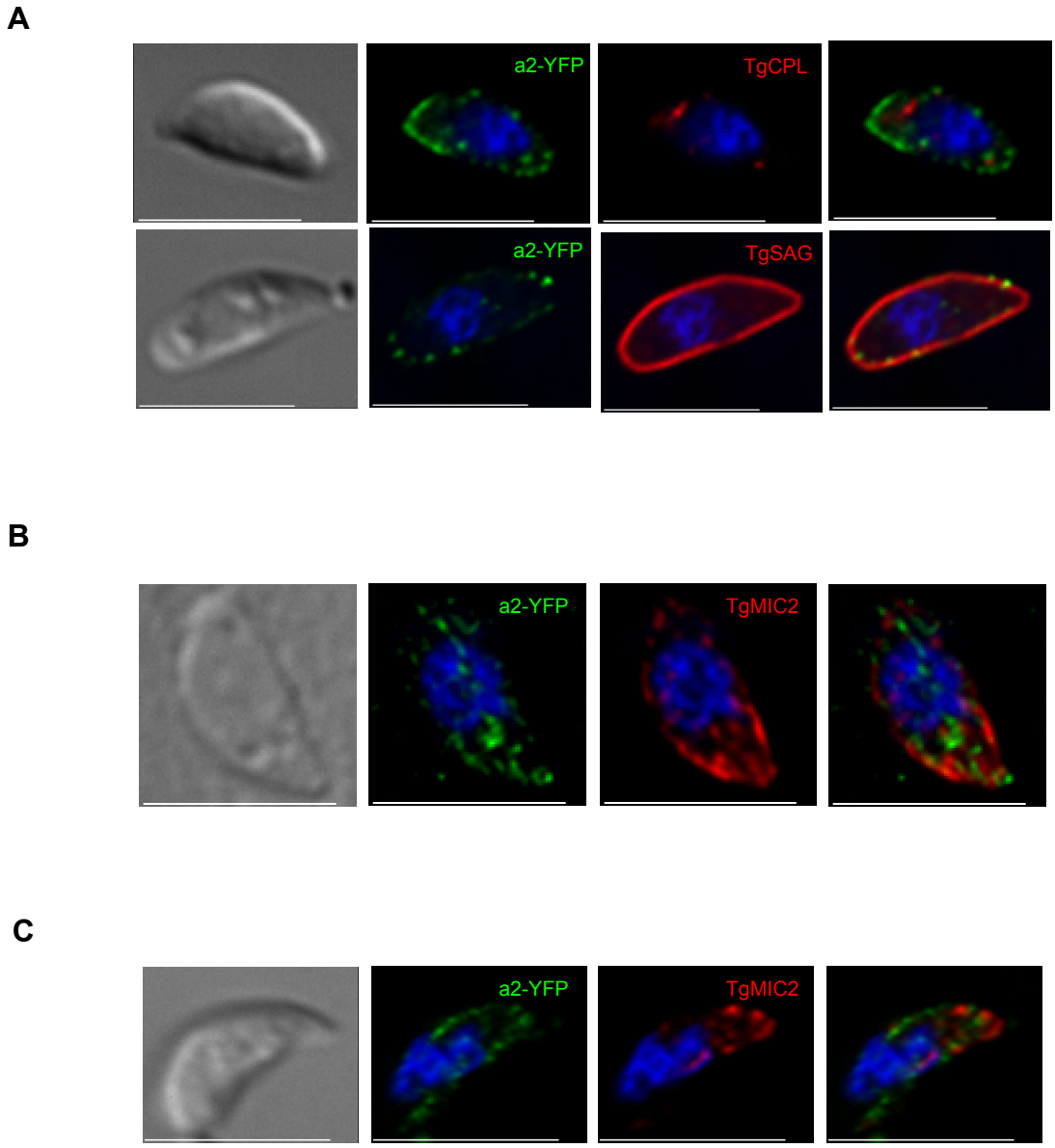


Figure 2.8 Localization of TgVHA-a2-YFP in extracellular and intracellular tachyzoites. (A) Extracellular parasites were collected and harvested in BAG for 30 min before fixing. (B) A representative invading tachyzoite before replication. (C) A representative recently egressed tachyzoite collected following a semisynchronization protocol (Fig 2.6). Labeling with anti-GFP (1:2000, green), anti-TgCPL (1:4000, red), anti-TgSAG1 (1:2000, red) or anti-TgMIC2 (1:1000, red) antibodies is shown. DAPI staining of the nucleus is shown (blue). Scale bars = 5 μm.

CHAPTER 3

CHARACTERIZATION OF THE *T. GONDII* V-H$^+$-ATPASE PROTON-TRANSLOCATING SUBUNITS

3.1 Objective

The main function of V-H$^+$-ATPases in plants and yeast is vacuolar acidification. We implemented a genetic approach to characterize the V-H$^+$-ATPase by knockout of the *a* subunit. Since *T. gondii* has two proton-translocating subunits, we hypothesized that it was possible to directly knockout one of the *a* subunits because the second one could complement its function. Thus, we attempted to directly knock out the *TgVHA-a2* gene to investigate its effect on the localization of TgVHA-a1 and determine if it is an essential gene. In addition, we overexpressed *TgVHA-a1* in a Δ*TgVP1* parasite line to test if an additional copy of *TgVHA-a1* could complement for the absence of the *TgVP1* gene. The rationale for these experiments were based on the assumption that both proton pumps function in vacuolar acidification. However, it is possible that the *T. gondii* V-H$^+$-ATPase is more important for PLV acidification than TgVP1 and we sought to test this hypothesis by overexpressing TgVHA-a1.

3.2 Materials and Methods

3.2.1 Cosmid-based strategy for TgVHA-a2 direct knockout

Since the gene size of *TgVHA-a2* was predicted to be almost 10 kb, with 14 exons, we envisioned using a *T. gondii* cosmid engineering strategy in order to knock out the endogenous gene. The cosmid TOXOW86 covers the *TgVHA-a2* gene and we introduced this cosmid into the EL250 strain of *E. coli* for cosmid recombineering, as described by

Brooks et al.[95] The cosmid library available from Dr. Striepen's laboratory includes 40-50 kb inserts of *T. gondii* wild-type RH genomic DNA and it is highly efficient in targeting the gene of interest because it provides large regions of homology that facilitate homologous recombination in the parasite. The cosmid strategy consists in modifying the cosmid by using a cassette generated by PCR using the pH3CG plasmid (kindly provided by Dr. Boris Striepen) as template and primers containing 50 bp flanking regions. The modification cassette contained the gentamicin resistance marker for bacterial selection and the chloramphenicol (CAT) resistance marker for selection of transgenic parasites (Fig 3.1). The parental cosmid was modified by homologous recombination in recombineering competent bacteria. Bacterial clones containing the modified cosmid were screened by digestion with restriction enzymes and PCR.

3.2.3 Overexpression of TgVHA-a1

An additional copy of the *TgVHA-a1* gene tagged with HA was introduced into the Δ*ku80*, TgVHA-a1-YFP and Δ*TgVP1* parasite lines, which were selected with 20 μM chloramphenicol. The Δ*TgVP1* parasite line was generated in our laboratory by Dr. Jing Liu, who kindly provided the cells for the overexpression studies of *TgVHA-a1*.

The open-reading frame of *TgVHA-a1* was inserted into the recombinant pCTH expression vector (kindly provided by Dr. Boris Striepen) between the *Bgl*II and *Avr*II restriction sites (see Appendix B for primer sequences). Insertion was confirmed by digestion with restriction enzymes and DNA sequencing. Twenty-five micrograms of recombinant plasmid were used to transfect *T. gondii* tachyzoites, which were inoculated in 12-well plates containing h-Tert fibroblasts grown in coverslips. Parasites were cultured for 16-20 h and used for immunofluorescence analysis.

3.3 Results

3.3.1 Direct knockout of *TgVHA-a2* reveals it may be an essential gene

A *T. gondii* parental cosmid was modified to delete *TgVHA-a2* and replace it with the *CAT* resistance gene (Fig 3.1B). Deletion of *TgVHA-a2* was confirmed in bacterial clone 1 by PCR (Fig 3.1D-E) and digestion with restriction enzymes (Fig3.1F). The modified cosmid (c1) was used to directly knockout *TgVHA-a2* in the Δ*ku80* parasite line. A large number of clones were screened but all of them still contained the *TgVHA-a2 gene*. This result suggests that *TgVHA-a2* may be an essential gene. Generation of conditional knockouts of *TgVHA-a2* remains to be performed to study the role of the V-H^+-ATPase in *T. gondii*.

3.3.2 Both TgVHA-a1-YFP and TgVHA-a2-YFP localize to vesicles at the plasma membrane of Δ*TgVP1* extracellular tachyzoites

To determine if TgVHA-a1-YFP and TgVHA-a2-YFP change their localization in the absence of the vacuolar proton pyrophosphatase TgVP1, the Δ*TgVP1* parasite line was transfected with the pYFP-LIC-DHFR vector containing the 3'-end of the gene of interest (Fig 3.2). These parasites were sorted by FACS and used for immunofluorescence. First, we observed that both *T. gondii a* subunits had a plasma membrane vesicular distribution and localized partially with the adhesin TgMIC2 (Fig 3.2). TgVHA-a1-YFP had a more apical distribution compared to TgVHA-a2. Both phenotypes were different in comparison to the Δ*ku80* line and the YFP-tagged original versions of the proteins, indicating a direct effect of the loss of TgVP1 in the localization of the *T. gondii a* subunits. It is possible that the *T. gondii* V-H^+-ATPase is translocated to the apical end and the plasma membrane by the secretory pathway where it could function to balance the intracellular pH of the parasite.

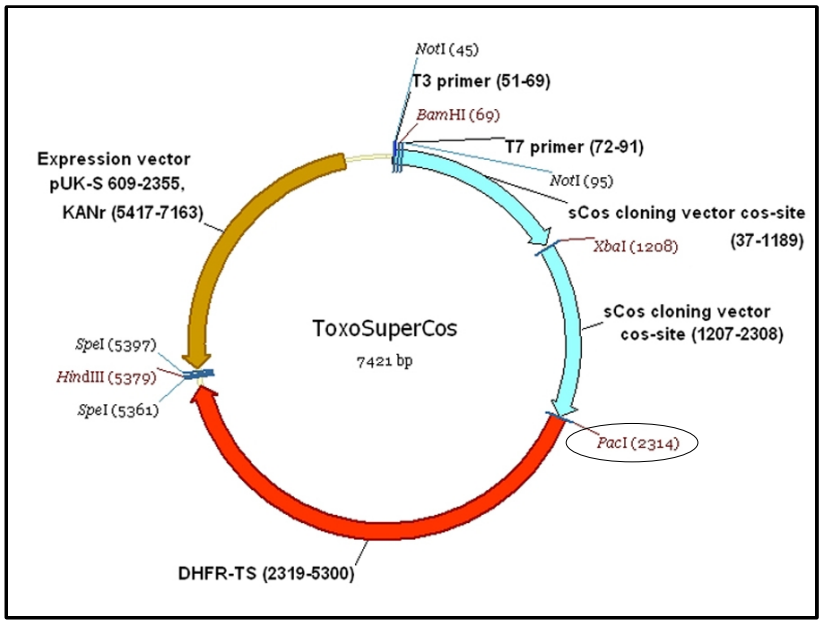
3.3.3 Overexpression of TgVHA-a1-HA restores the appearance of TgCPL inside a compartment in Δ*TgVP1* extracellular tachyzoites

No difference in endogenous TgVHA-a1-YFP expression was detected between Δ*ku80* and Δ*TgVP1* tachyzoites (Fig 3.2A, *right panel*), suggesting that endogenous expression levels of TgVHA-a1 are not sufficient to compensate for the loss of the *TgVP1* gene. The *TgVHA-a1* gene was overexpressed in Δ*ku80*, TgVHA-a1-YFP and Δ*TgVP1* parasite lines to determine the effect on extracellular tachyzoites and investigate if overexpression of *TgVHA-a1* could complement *TgVP1* in the Δ*TgVP1* parasite line. *TgVHA-a1* was tagged with the HA epitope and transfected in *T. gondii*. Figure 3.3 shows immunofluorescence of transient transfections. Overexpression of *TgVHA-a1-HA* showed that TgVP1 localizes in a ring-like compartment close to the nucleus in the Δ*ku80* line (Fig 3.3A). TgVHA-a1-HA also localized closed to the nucleus in extracellular tachyzoites, probably the endoplasmic reticulum (ER), suggesting that it is retained there as a result of the overexpression. No clear localization of TgVHA-a1-YFP to the PLV was observed in extracellular parasites overexpressing *TgVHA-a1-HA* (Fig 3.3A), indicating that the localization of TgVHA-a1 is affected due to an imbalance in the intracellular pH of extracellular tachyzoites.

The Δ*TgVP1* line displayed a characteristic difference in the localization of the cathepsin TgCPL (Fig 3.3B). Immunofluorescence staining using anti-TgCPL antibody showed that TgCPL localizes to vesicles at the apical site where the PLV is usually found. The vesicle localization may indicate that the protease is not able to mature due to a change in the luminal pH of the PLV. Alkalinization of intracellular organelles inactivates acidic proteases like TgCPL, and it may explain why it localizes to vesicles inside the PLV when TgVP1 is absent. TgCPL

was also observed in vesicles inside the PLV in the TgVHA-a1-YFP parasite line (Fig 3.3B). These vesicles could be acidocalcisomes containing the V-H^+-ATPase, as we previously demonstrated[9]. However, when *TgVHA-a1-HA* was overexpessed in the Δ*TgVP1* line, we observed a ring-like structure labeled with the anti-TgCPL antibody positioned close to the nucleus of extracellular tachyzoites (Fig 3.3B). This compartment seems to localize to the endoplasmic reticulum or Golgi apparatus because of its vicinity to the nucleus. These data suggest that the overexpressed TgVHA-a1-HA is retained in the ER, causing an accumulation of TgCPL in an intracellular compartment and disruption of TgVHA-a1 protein trafficking to the PLV. Colocalization studies with ER and Golgi markers will confirm these results.

A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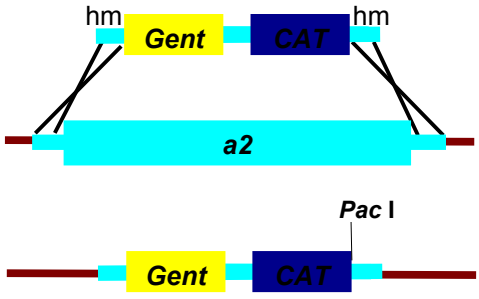
sCos cloning vector
cos-site (1207-2308)
PacI (2314)
Expression vector
pUK-S 609-2355,
KANr (5417-7163)
ToxoSuperCos
7421 bp
SpeI (5397)
HindIII (5379)
SpeI (5361)
DHFR-TS (2319-5300)

B


hm
Gent
CAT
hm
a2
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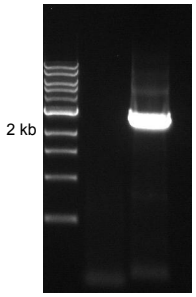
CAT

C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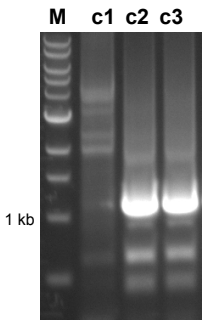
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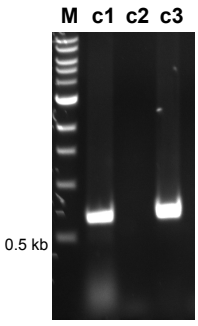
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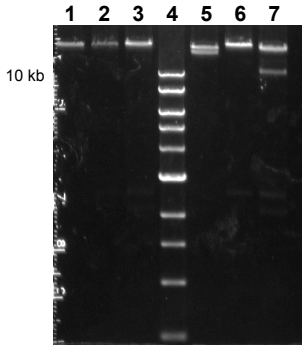
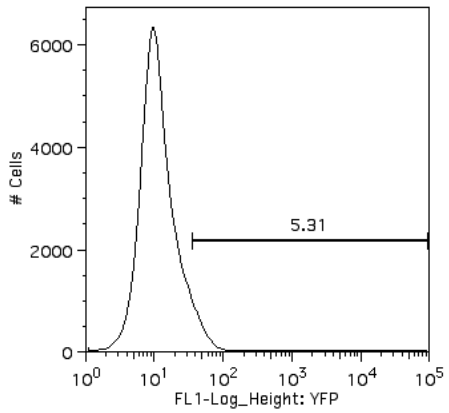
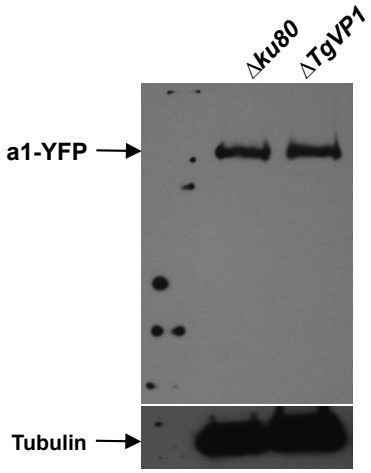

1 2 3 4 5 6 7
10 kb

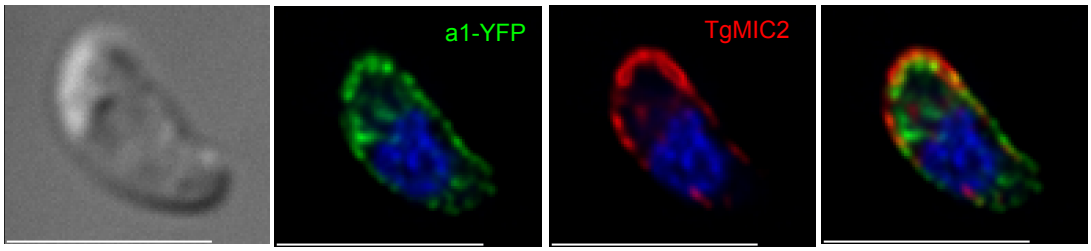
Figure 3.1 Cosmid recombineering strategy. (A) ToxoSuperCos parental cosmid containing large regions of *T. gondii* RH gDNA. *Pac*I is present in the parental cosmid *(encircled)*. (B) Graphic illustration of homologous recombination in the *TgVHA-a2* locus in the cosmid and replacement of the gene with the resistance marker cassette containing 50 bp flanking regions (hm). A *Pac*I site is also present in the modified cosmid next to the CAT resistance marker site. (C) PCR using as template the pH3CG plasmid to generate the recombination cassette (~2.1 kb) with primers containing 50 bp flanking regions. Three bacterial clones (c1-c3) were screened by PCR to confirm integration of the marker cassette into the cosmid (D-E). (D) PCR to confirm the deletion of *TgVHA-a2* in the modified cosmid using gene-specific primers (1 kb band size obtained when *TgVHA-a2* was present). (E) PCR to confirm presence of *CAT* gene (~0.6 kb) in modified cosmid using *CAT*-specific primers. (F) Digestions of parental *(left)* and modified *(right)* cosmids using a combination of restriction enzymes. Lanes 1-3 show digestions in the parental cosmid using *Pac*I (lane 1), *Xma*I (lane 2), *Pac*I/*Xma*I (lane 3). Lanes 5-7 show digestions in the modified cosmid (c1) using *Pac*I (lane 5), *Xma*I (lane 6), *Pac*I/*Xma*I (lane 7). Lanes M in (D) and (E) and lane 4 in (F) show the 1 kb DNA ladder (New England Biolabs).

A





ΔTgVP1 / a1-YFP



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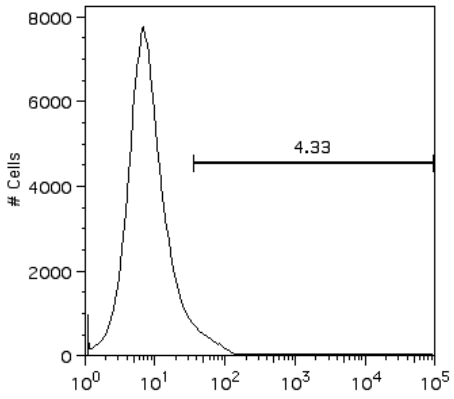


ΔTgVP1 / a2-YFP

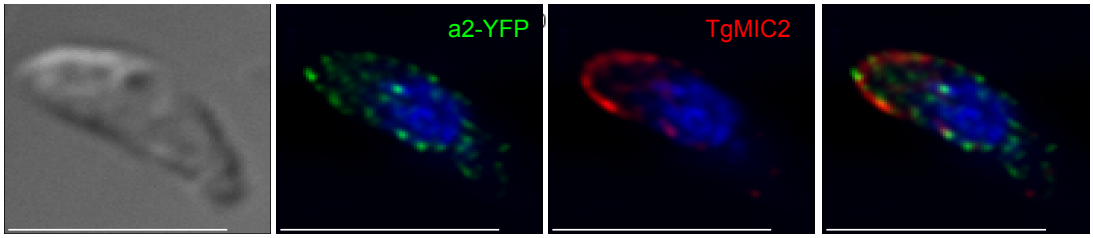
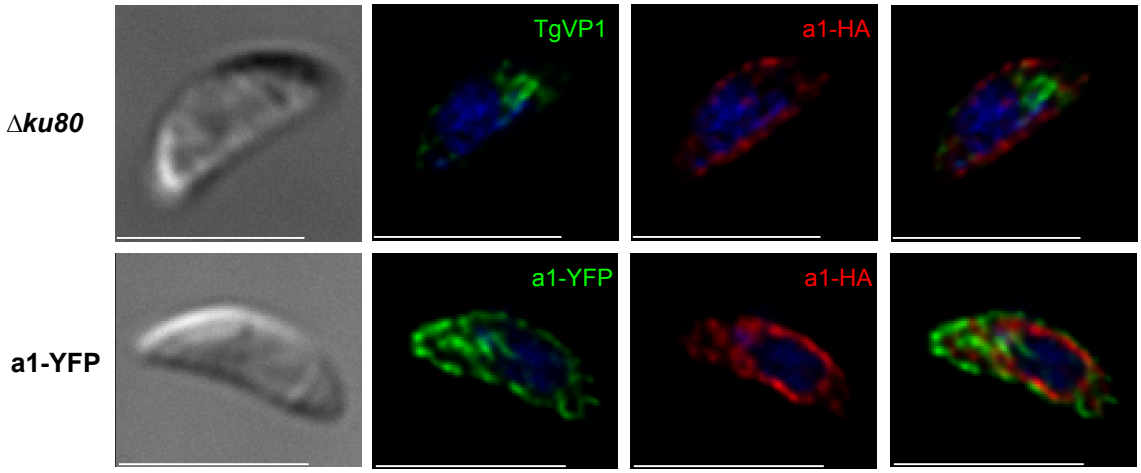


Figure 3.2 Localization of TgVHA-a1-YFP (A) and TgVHA-a2-YFP (B) in Δ*TgVP1* extracellular tachyzoites. Expression of TgVHA-a1-YFP was detected by Western blot (A, *right panel*). Parasites were sorted by FACS (A, *left* panel and B, *top panel*) and used for indirect immunofluorescence (A and B, *bottom panels*). Primary antibodies against GFP (1:2000, *green*) and TgMIC2 (1:1000, *red*) were used for localization. Commercial secondary antibodies were used at 1:1000 dilution. DAPI staining of the nucleus is shown (blue). Scale bars = 5 μm.

A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TgVP1
a1-HA
a1-YFP
a1-YFP
a1-HA

B

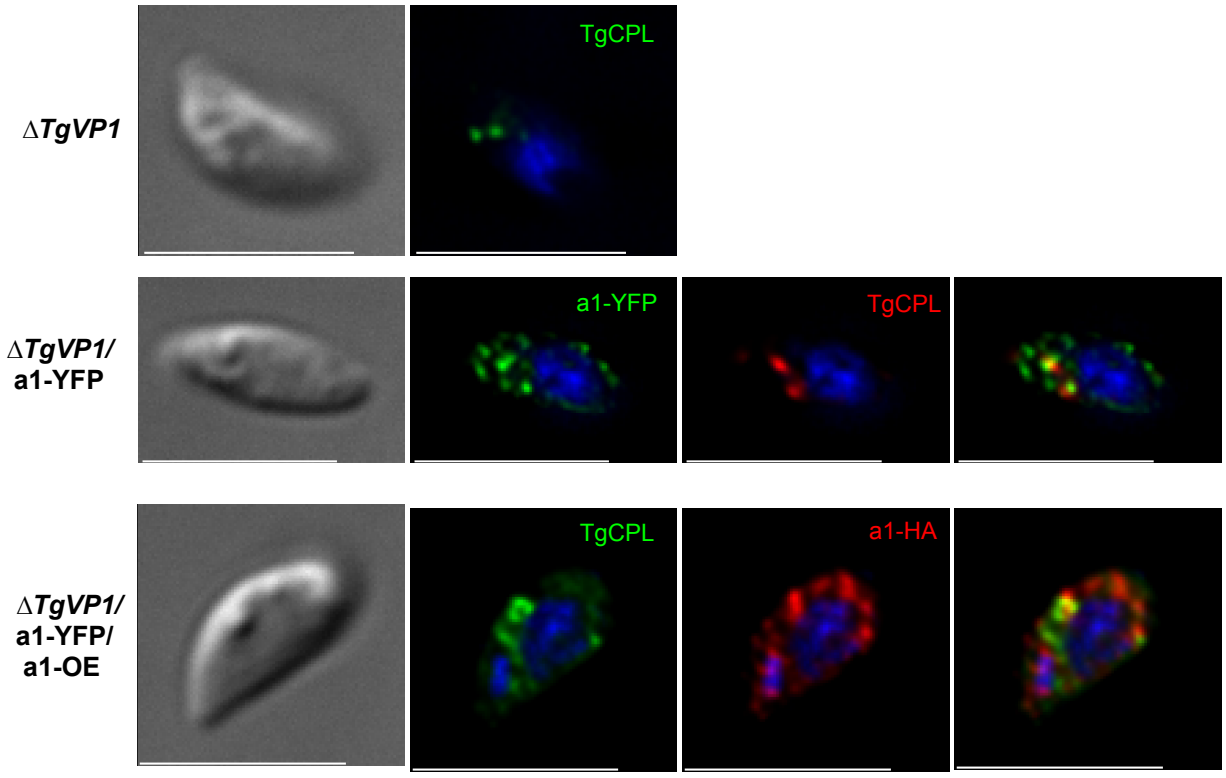
ΔTgVP1
TgCPL
ΔTgVP1/
a1-YFP
a1-YFP
TgCPL
ΔTgVP1/
a1-YFP/
a1-OE
TgCPL
a1-HA

Figure 3.3 Overexpression of TgVHA-a1-HA in extracellular tachyzoites. A parasite transient transfection with the pCTH expression vector containing *TgVHA-a1* in frame to *HA* was done to observe the localization in *Δku80* (A, top panel), TgVHA-a1-YFP (A, bottom panel) and *ΔTgVP1* (B) parasite lines. Primary antibodies against rabbit TgVP1 (1:4000, green), TgCPL (1:4000, green or red) and GFP (1:2000, green) were used together with the HA tag (1:200, red) antibody. Commercial secondary antibodies (Molecular Probes) were used at 1:1000 dilution. DAPI staining of the nucleus is shown (blue). Scale bars = 5 μm.

CHAPTER 4

DISCUSSION

This work presents the characterization of two proton-translocating subunits of the *T. gondii* V-H^+-ATPase. The localization of subunits TgVHA-a1 and TgVHA-a2 suggest they play a role in the secretory pathway of the parasite, as TgVHA-a1 localizes to the plant-like vacuole (PLV), and the plasma membrane in vesicle-like structures, whereas TgVHA-a2 is found in vesicles at the plasma membrane. The localization of TgVHA-a1 to the plasma membrane was demonstrated in intracellular and recently egressed tachyzoites. Extracellular tachyzoites incubated with BAG for at least 30 min displayed a localization to the PLV. It is evident that this drastic change in localization of TgVHA-a1 to the PLV is important for the extracellular stage, and it may have to do with our proposed role of the PLV in protection against environmental stress[9].

An intriguing aspect of this research is the localization of TgVHA-a1 to the plasma membrane in intracellular parasites after replication. In addition, TgVHA-a1 does not localize to the endosome-like compartment in which we previously showed the presence of TgVP1 in dividing tachyzoites[9]. TgVHA-a1 appears to be translocated from the PLV to the plasma membrane in intracellular tachyzoites after parasite replication. An interesting question is whether or not TgVHA-a1 is functional in the intracellular tachyzoite stage.

The localization of TgVHA-a1-YFP in the *ΔTgVP1* line shows that vesicles containing the TgV-H^+-ATPase accumulate at the apical end of extracellular tachyzoites. These vesicles colocalized partially with the anti-TgMIC2 antibody. TgMIC2 is proteolytically cleaved at the

C-terminus by rhomboid proteases[96], which are intramembrane serine proteases localizing at the membrane of intracellular tachyzoites[97]. The secretion of adhesins released from micronemes is a process required for host cell attachment, and the subsequent shedding of adhesins from the cell surface is important for parasite motility and invasion[94]. The localization of TgVHA-a1 suggests that the *T. gondii* V-H+-ATPase could be involved in vesicle trafficking from the PLV to the plasma membrane. Sorting of microneme proteins may occur from the PLV to microneme organelles in vesicles containing the *T. gondii* V-H+-ATPase. This could explain the observed changes in the localization of the *a* subunits in extracellular tachyzoites. It is also possible that the vesicles observed are acidocalcisomes, which can be confirmed by electron microscopy.

The localization studies of TgVHA-a2 revealed its association with vesicles at the surface and to the apical end of the parasite. TgVHA-a2 did not localize to the PLV but similarly to TgVHA-a1, it colocalized partially with the surface adhesin TgMIC2 in the *ΔTgVP1* parasite line. When we attempted to knockout the *TgVHA-a2* gene, no mutant parasites were obtained, suggesting that the gene is essential. Based on the localization of the *T. gondii* V-H+-ATPase *a* subunits with TgMIC2, it is possible that vesicles containing the V-H+-ATPase also transport TgMIC2 to its destiny at the apical end of the parasite. The fact that we could see colocalization of TgVHA-a2 with TgMIC2 in invading and recently egressed tachyzoites supports the idea that TgVHA-a2 could be involved in protein sorting and this could explain why the gene may be essential for the extracellular tachyzoite stage. In addition, when drastic changes in the intracellular pH occur, the V-H+-ATPase trafficking to the plasma membrane could be required for intracellular pH regulation through proton-pumping by the *a* subunit. The creation of subunit *a* conditional knockouts and future proton extrusion experiments will help clarify these unanswered questions.

Vesicles labeled with anti-TgCPL antibody were usually observed in Δ*TgVP1* parasites. The appearance of TgCPL in these vesicles was distinct from its characteristic localization observed in wild-type parasites.[9-10] The lack of V-H$^+$-PPase activity might lead to alkalinization of the PLV and consequent TgCPL compartmentalization in small vesicles. These vesicles containing TgCPL are probably more acidic (due to the presence of the V-H$^+$-ATPase) than the lumen of the PLV and could be a way to keep the function of the protease in a compartmentalized acidic environment. This data also indicates that endogenous levels of TgVHA-a1 and TgVHA-a2 are not sufficient to overcome the effects from the loss of TgVP1. However, when we overexpressed TgVHA-a1 in the *ΔTgVP1* line, TgCPL accumulation in an intracellular compartment was observed close to the nucleus. In addition, the overexpressed TgVHA-a1 protein localized to large vesicles close to the nucleus and to the site of TgCPL accumulation. It is possible that protein trafficking to the PLV was disrupted as a result of TgVHA-a1 overexpression, leading to accumulation of TgCPL because of the retention of the V-H$^+$-ATPase complex in the ER and/or Golgi apparatus. The retention of the V-H$^+$-ATPase complex in the ER could have a devastating effect on extracellular tachyzoites as they would not be able to regulate the intracellular pH. Future studies on the functional roles of both the *T. gondii* V-H$^+$-PPase and V-H$^+$-ATPase in the PLV will help elucidate the pH regulatory mechanisms driven by these enzymes in extracellular tachyzoites.

CHAPTER 5

CONCLUSION

The *T. gondii* V-H+-ATPase is an important component of the secretory pathway of the parasite. Two functional roles are predicted from our findings: functions in (1) PLV acidification and (2) vesicle trafficking from the PLV to the plasma membrane. It is possible that the primary function of TgVHA-a1 is vacuolar acidification, whereas TgvHA-a2 is involved in protein sorting through the secretory pathway. This research has implications for the study of V-H+-ATPases in apicomplexan parasites and provides evidence for the importance of the enzyme in key regulatory processes for the survival of the extracellular tachyzoite stage.

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APPENDIX A

Table A1: V-H$^+$-ATPase subunits found in *T. gondii*

V-ATPase subunit	Gene ID (ToxoDB)	Spectrum Count Ratio VP1-OE (F1:P3)	Tachyzoite Differential Expression	Identity in Yeast (%)	Function in Yeast
TgVHA-a1	**TGME49_032830**	**8.7**	**9.11**	**27**	**essential for assembly and catalytic activity;**
TgVHA-a2	**TGME49_090720**	**NO**	**7.2**	**26**	**Arg in TM7 is essential for proton translocation**
TgVHA-B	TGME49_019800	++	5.64	73	3 non-catalytic sites which interact with three peripheral stalks (EG subunits)
TgVHA-A	TGME49_056970	2.1	7.62	57	3 catalytic sites; ATP hydrolysis
TgVHA-c'	TGME49_012310	1.8	11.11	52	proteolipid subunit; functions in vacuolar
TgVHA-c'''	TGME49_023250	NO	5.17	41	acidification; Glu in TM 3 or 4 essential in proton
TgVHA-c''	TGME49_091310	NO	7.2	46	translocation
TgVHA-d	TGME49_059010	2.2	7	34	stabilizes V0 subunits; required for V1 domain assembly (DF central stalk)
TgVHA-e	TGME49_051470	NO	7.62	NO	essential for vacuolar acidification; interacts with assembly factor Vma21p in the ER; involved in V0 biogenesis
TgVHA-C	TGME49_115620	NO	2.97	25	required for the V1 domain assembly onto the vacuolar membrane
TgVHA-D	TGME49_081920	NO	5.46	36	plays a role in the coupling of proton transport and ATP hydrolysis
TgVHA-E	TGME49_105290	++	5.22	31	required for the V1 domain to assemble onto the vacuolar membrane
TgVHA-F	TGME49_110960	NO	8.6	51	required for the V1 domain to assemble onto the vacuolar membrane
TgVHA-G	TGME49_046560	++	10.37	NO	required for the V1 domain to assemble onto the vacuolar membrane
TgVHA-H	TGME49_008590	NO	7.18	24	serves as an activator or a structural stabilizer of the V-H+-ATPase

APPENDIX B

Table B1: Nucleotide sequences of primers used in this study

Gene sequencing analysis	
TgVHA-a1 F1	5'-ATGACCACTCTACGCAGCG-3'
TgVHA-a1 Stop	5'-CTACGCATCATCCTCGCCTTG-3'
TgVHA-a1 F1972	5'-GCCGAGGTCAAGAGCGAAG-3'
TgVHA-a1 R2355	5'-CGTGCCAAGAATGAACTCGATCG-3'
TgVHA-a2 F1	5'-ATGGGGATATTTCGGTCGGAG-3'
TgVHA-a2 Stop	5'-TTAAGATCCTTGAGGTCTCGACTCTC-3'
TgVHA-a2 F1317	5'-GAGAAGGTGATTCGTTTGTGCG-3'
TgVHA-a2 F2157	5'-GATCAACGTCCTCATCGACATGC-3'
TgVHA-a2 R 2683	5'-TTTGGTGGATGAAGACTTCGCTG-3'
TgVHA-a2 R2325	5'-GGAAGAATGCCACAATGACAGAGAAC-3'
TgVHA-a2 R551	5'-CTCCGCAAACAGAAAACGCAAA-3'
TgVHA-a2gDNA-Up2914160	5'-CGATTCTACCGCCAGTTACTCTTCTTG-3'
TgVHA-a2gDNA-Up2914700	5'-GCGAAGGAAAGCACAGAGGAC-3'
Generation of LIC fragments	
TgVHA-a1 F-LIC	5'-TACTTCCAATCCAATTTAATGCCGTCGCAGGTCAT-3'
TgVHA-a1 R-LIC	5'-TCCTCCACTTCCAATTTTAGCCGCATCATCCTCGCCTTG-3'
TgVHA-a2 F-LIC	5'-TACTTCCAATCCAATTTAATGCGTGGTTCTGCTGTCTGTGCC-3'
TgVHA-a2 R-LIC	5'-TCCTCCACTTCCAATTTTAGCAGATCCTTGAGGTCTCGACTCTC-3'
TgVHA-a2 knockout screening	
TgVHAa2 F1-56727	5'-CTGTCATTGTAAGGAGAGAGCG-3'
TgVHAa2 R2-57553	5'-GGCAGACTGGGGATTACTTGC-3'
Overexpression of TgVHA-a1	
TgVHA-a1 *Bgl*II	5'-gaagatctATGACCACTCTACGCAGCG-3'
TgVHA-a1 *Avr*II	5'-cgcctaggCGCATCATCCTCGCCTTG-3'
Screening for YFP integration	
LA015-YFPLIC-R (200 bp)	5'-CTCGCCCTCGCCGGACACGC-3'
YFP-R (entire gene)	5'-CTTGTACAGCTCGTCCATGCCG-3'
Screening for CAT resistance marker	
F-CAT	5'-ATGCATGAGAAAAAAATCACTGGATATACC-3'
R-CAT	5'-ACTCATCGCAGTACTGTTGTAATTC-3'