IDENTIFICATION AND ASSESSMENT OF GENETIC VACCINE CANDIDATES FROM *TRYPANOSOMA CRUZI*

by

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(Under the Direction of Rick L. Tarleton)

ABSTRACT

Genetic vaccination has been shown to be an effective means for controlling *Trypanosoma cruzi* infection and development of Chagas disease, yet to date only six genes have been tested including five members of the trans-sialidase (TS) gene family. Vaccination with these genes generates an immune response able to significantly control the infection, however parasite clearance was not attained and in each study a small percentage of the animals did not survive. The goal of this study was to expand the diversity of genes assessed as genetic vaccine candidates from *T. cruzi*. Three non-TS genes were assessed as genetic vaccines, Tcβ3, FCaBP, and LYT1. Although peptides from all three genes were found to be targets of cytotoxic T cell responses in chronically infected mice, cells that are critical for control of *T. cruzi* infection, only immunization with *LYT1* protected mice from a normally lethal challenge of *T. cruzi*. As an alternative to testing individual *T. cruzi* genes as vaccines, pools of genes from the TS and mucin families were assessed in vaccination studies. The mucin family was selected because like the TS family it encodes GPI-anchored surface proteins that are abundantly

expressed. We found that immunization with pools of TS but not mucin genes provided protection against a normally lethal challenge of *T. cruzi*.

Although Tcβ3 was not effective as a genetic vaccine, its homology to the human β3 subunit of the AP-3 adaptor protein complex suggested it might play a role in protein trafficking in *T. cruzi* and prompted further investigation. No other adaptin molecules have been identified in *T. cruzi* and very little is known about protein trafficking in this organism. Unable to generate null mutants of Tcβ3 in *T. cruzi*, we used its sequence to identify its homologue from *T. brucei*, Tbβ3, and took advantage of the inducible RNAi expression system developed in *T. brucei* to express dsRNA for this gene. Parasites expressing dsRNA for Tbβ3 were unable to complete cytokinesis and exhibited defects in flagellar adhesion.

INDEX WORDS: *Trypanosoma cruzi*, Chagas disease, Genetic vaccination, DNA immunization, β3 adaptin, AP-3, Adaptor protein complex

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To Greg

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

T. cruzi infection and Chagas disease

Chagas disease is the most serious parasitic disease in Latin America and is amongst its leading health problems. The protozoan parasite, *Trypanosoma cruzi*, is the causative agent of this disease which claims the lives of fifty thousand people each year. A total of 16-18 million people are currently infected and another 100 million are at risk of infection throughout Central and South America. [1, 2] The acute stage is characterized by systemic parasitemia and is followed by a virtually asymptomatic stage that can last for several decades. Eventually, approximately one third of those chronically infected begin to manifest the symptoms of Chagas disease, developing severe cardiac and/or gastrointestinal disease, which in most cases leads to death. There is no cure for Chagas disease but two chemotherapeutic agents, nifurtimox and benznidazole, can be effective to treat infection with *T. cruzi*. However, the availability and use of these drugs is limited due to their extreme toxicity.

The concentration of Chagas disease in Latin America can be attributed to the fact that vector-borne transmission is the primary mode of transmission and accounts for 90% of human infections. Even though the geographic distribution of the parasite's natural vector, the reduviid bug, stretches far into the middle regions of the United States, it is only in Latin America that a few species have become adapted to the domestic

environment, living in the cracks of rural housing and surviving on the blood of their vertebrate hosts, including humans [3, 4].

Unlike other trypanosomatids, *T. cruzi* is not transmitted via the saliva secreted during the blood meal but rather in the feces released after the blood meal is taken. The infective stage of the parasite, metacyclic trypomastigotes, must then transverse the skin through a wound (possibly the bite wound itself) or a mucosal surface to establish an infection. Nonreplicative metacyclic trypomastigotes can invade a wide variety of cell types by inducing the recruitment and fusion of lysosomes with the host cell plasma membrane (reviewed in [5]). The parasite enters the cell via this acidic phagolysosomal compartment from which it subsequently escapes. A secreted acid-stable hemolytic protein, TcTox, is thought to play a role in the breakdown of the phagolysosome membrane resulting in the release of the parasite into the cytosol [6-9]. In the cytosol the trypomastigote transforms into the replicative amastigote stage. After several rounds of replication by binary fission amastigotes transform into trypomastigotes, which eventually break out of the cell and then can infect neighboring cells or spread to other tissues through bodily fluids like the blood. It is the parasite's presence in the blood where they are available for ingestion by a blood feeding reduviid that completes the life cycle.

In addition to vector-borne transmission, *T. cruzi* can also be transmitted by blood transfusion, organ transplantation and transplacentally or orally by ingestion of contaminated material. Transmission by blood transfusion has become a large problem in Latin America and is becoming a concern for the United States. Several million people from endemic countries have emigrated to the U.S. over the last several decades and it

has been determined that in certain areas of the U.S. 1 in 7,800 blood donors are seropositive for *T. cruzi* and the overall seroprevelence rate for the U.S. blood donors is 1 in 35,000. Five cases of transmission of *T. cruzi* through blood transfusion have been documented in North America yet the U.S. is still not screening the blood supply for *T. cruzi* [10-12].

Three multi-government initiatives to eliminate Chagas disease, between countries in the Southern Cone, the Andean region, and in Central America, have made tremendous progress in decreasing the levels of vector and blood-borne transmission of *T. cruzi* [13]. Transmission of Chagas disease has been virtually eliminated in both Uruguay and Chile [3, 14] and the number of newly reported annual cases of Chagas disease in all of Latin America has dropped from 700,000 in 1990 to 200,000 in 2000 [13]. These reductions are in large part due to vector control and blood bank and donor screening programs.

Vector control programs have been very effective yet they are difficult to coordinate, expensive to maintain and can lead to the development of insecticide resistance. In addition, as *T. cruzi* prevalence rates drop these programs may become less of a priority. Consequently there must be continued efforts towards the development of better treatments and an effective vaccine for *T. cruzi.*

Chagas disease etiology

For many years, vaccine development for *T. cruzi* was controversial due to the fact that Chagas disease was thought to be an autoimmune phenomena in which the immune system and not parasite presence was thought to be causing disease. This belief

has not only stunted efforts and funding for vaccine development, as evidenced by the absence of any international vaccine initiatives, but has also discouraged the therapeutic treatment of Chagasic patients. The autoimmune hypothesis is founded on the presence of anti-self antibodies and lymphocytes as well as the difficulty of detecting parasites in the tissues of chronically infected individuals (reviewed in [15]). However the mere existence of a detectable anti-self response is not experimental evidence supporting this as the source of disease development. Furthermore a clear connection between sites of disease and the presence of parasites has been established with the advent of more sensitive techniques such as immunohistochemistry, *in situ* PCR and *in situ* hybridization (reviewed in [16]). The spectral nature of disease development in both mouse and human *T. cruzi* infections, ranging from the absence of disease to development of disease in a variety of muscle tissues, has enabled further confirmation of the correlation between parasite persistence and disease development. For example, in mice/parasite strain combinations that lead to disease development predominantly in the skeletal muscle, parasite DNA is only detected in the skeletal muscle and not in heart tissue. Whereas in mice/parasite strain combinations that lead to cardiomyopathy, parasite DNA is detected in heart and not skeletal muscle [17]. Likewise parasite DNA has been found in heart tissue from human cadavers that had previously been diagnosed with Chagasic cardiomyopathy and not in heart tissue from seropositive cadavers without evidence of heart disease [18]. A similar correlation has been found in human oesophageal tissue [19]. These data provide solid evidence that parasites do persist at sites of disease.

Immunity to *T. cruzi*

Immune control of *T. cruzi* in the mammalian host is evidenced by the resolution of the systemic parasitemia present during acute stage of infection. Although the tissue and blood parasite burden is dramatically reduced it is not completely abolished. This has been demonstrated by the detection of parasites in the tissues of chronically infected mice and humans, as described above, as well as by the deleterious effects of immuno-suppression. Reactivation of infection has been documented in association with treatment with immunosuppressive drugs [20, 21] and in HIV patients, whose immune systems are compromised as a result of viral infection [22-28]. Studies utilizing mice lacking specific immune effector functions, as a result of genetic manipulation or mAb treatment, indicate that both humoral and cell-mediated immune components are essential for the control of *T. cruzi* infection (reviewed in [15]). As humoral immunity, which is mediated by antibodies, is most effective in controlling extracellular pathogens and cell mediated immunity is important for the control of intracellular pathogens, it is not surprising that both types of immunity are required to fight a pathogen exhibiting both intra- and extracellular stages. The knowledge that the immune system is capable of controlling parasitemia in combination with the fact that only 30% of those infected develop disease suggests that if the immune response against *T. cruzi* were enhanced, disease may be reduced and possibly eliminated.

Humoral immunity. The ability of antibody to mediate control of *T. cruzi* infection is demonstrated by the protection against lethal infection that is conferred by the passive transfer of immune serum from *T. cruzi* infected mice [29, 30]. Furthermore, pretreatment or co-injection of *T. cruzi* with anti-*T. cruzi* antibodies leads to the rapid

clearance of parasites [31, 32] and mice with deficiencies in antibody production are more susceptible to *T. cruzi* infection than immunologically normal mice [33, 34]. Studies have demonstrated that the antibody dependent protection against *T. cruzi* infection is mediated by opsonic [31], lytic [35], antibody dependent cellular cytotoxicity [36], complement activating [37-39] and invasion blocking activities [40] of anti-*T. cruzi* antibodies.

Cell mediated immunity. The critical role that both $CD4^+$ and $CD8^+$ T lymphocytes play in the control of *T. cruzi* infection has been demonstrated by the susceptibility of mice that have deficiencies in either of these cell types due to genetic manipulation or antibody treatment [41-46]. Mice deficient in either subset exhibit high blood and tissue parasite levels and do not survive the acute stage of infection. Significantly higher parasite burdens and earlier mortality is seen in mice lacking both $CD4^+$ and $CD8^+$ T cells indicating that these subsets have distinct roles in protection against *T. cruzi* infection [44].

The primary effector function of $CD4^+$ T cells, also known as T helper cells, is the production of cytokines that can activate many cells including B cells (leading to antibody production), macrophages, other T cells and inflammatory cells. The importance of $CD4^+$ T cells for the activation of such a wide variety of immune effector cells elucidates the significance of their loss for the control of *T. cruzi* infection. Two lineages of helper T cells have been characterized based on the cytokines they produce. Th1 cells are typified by IFN-γ production and Th2 cells by IL-4 production. Type 1 responses have been shown to be critical for control of other intracellular pathogens and it has recently been demonstrated that a type 1 response is protective in *T. cruzi* infection

[47, 48]. Mice that can not generate a type 1 T helper cell response due to a defect in the Stat 4 gene, a transcriptional regulator required for responsiveness to IL-12 which drives Th1 cell development, are extremely susceptible to *T. cruzi* infection [48]. However mice lacking the ability to generate Th2 cells, because of a defect in the Stat6 gene required for responsiveness to IL-4 which drives Th2 cell development, exhibit parasite levels and longevity comparable to wild-type mice. Furthermore, mice receiving Th1 cells specific for a parasite antigen protected mice against *T. cruzi* induced mortality whereas the transfer of Th2 cells specific for the same antigen was not protective [47]. These results corroborate data indicating that IFN-γ is critical for control of *T. cruzi.* Mice treated with anti-IFN-γ antibodies [49-52] and mice that are genetically incapable of responding to IFN-γ are extremely susceptible to *T. cruzi* infection [53] whereas the administration of IFN-γ enhances parasite clearance [54, 55].

As described above $CD8^+$ T cells are important for control of *T. cruzi* infection. Although it was demonstrated several years ago that $CD8^+$ T cells from *T. cruzi* infected mice were capable of lysing *T. cruzi* infected target cells, it is only recently that specific *T. cruzi* $CD8^+$ T cell peptide epitopes have been identified [56-58]. Furthermore it has been demonstrated that $CD8^+$ T cells specific for a peptide epitope from TSA-1, a *T. cruzi* trans-sialidase-like molecule, have been shown to transfer protection to naïve mice [56]. These cells were highly cytolytic and potent producers of IFN-γ. $CD8^+$ T cells can mediate immune control through several mechanisms including the induction of cell lysis by perforin/granzyme release, by Fas/FasL interactions or by cytokine production. The $CD8^+$ effector mechanism involved in control of *T. cruzi* infection has been investigated. It has been demonstrated that mice deficient in either perforin or granzyme B are not

more susceptible to *T. cruzi* infection than wild-type mice indicating that the $CD8^+$ T cell effector mechanism important in controlling *T. cruzi* infection is not dependent on perforin/granzyme mediated cytolysis [34].

In summary it has been demonstrated that antibody, a type 1 $CD4^+$ T cell response, and $CD8^+$ T cell function are critical components of an effective anti-*T. cruzi* immune response. However there are many unanswered questions. Why the immune response is capable of clearing parasites from most tissues except muscle and why a subset of chronically infected people develop disease while the majority do not are among the most intriguing questions whose answers may lead the way to curing Chagas disease.

Vaccination in *T. cruzi*

Vaccination efforts in *T. cruzi* have been thwarted by the fear of vaccine-induced disease exacerbation and by the focus on generating protective antibody responses against the trypomastigote stage. Now that it has been clearly demonstrated that parasite persistence, not autoimmunity, is the causative agent of Chagas disease and that a complex immune response, not just antibody, is required to control *T. cruzi* infection, progress towards an effective *T. cruzi* vaccine can be made. Conventional vaccination strategies, including immunization with live attenuated or non-proliferative organisms, killed intact parasites, purified protein or cell homogenates have not been effective against *T. cruzi* infection. These vaccination strategies are effective at generating antibody responses but are not as effective at stimulating $CD8^+$ T cell responses which are critical for control of *T. cruzi* infection (described above). In contrast, genetic

vaccination has been shown to generate both humoral and cell mediated immune responses (reviewed in [59]). These vaccines usually consist of a plasmid vector capable of replicating in *E. coli* and driving protein expression in mammalian cells via a strong promoter [59, 60]. The gene of interest is cloned into a mammalian expression plasmid, which is cultivated in bacteria, purified and is ready for injection [59, 60]. Recently, a number of studies have demonstrated the potential of genetic vaccination as a means to protect against *T. cruzi* infection in the murine model [61-67]. Five of the six genes used in these studies are members of the large trans-sialidase (TS) gene family, a family consisting of approximately 1000 members, a majority of which are not enzymatically active [68-70]. Genetic vaccination with the TS family members induced antibody production, elicited a cytotoxic $CD8^+$ T cell response, two things that are critical for control of *T. cruzi* infection, and provided significant protection against *T. cruzi* infection that was $CD8^+$ and $CD4^+$ T cell dependent. This protection was amplified when cytokine-encoding plasmids were co-administered. Not only did most of the mice survive a normally lethal infective dose of the parasite but they also demonstrated a significant reduction in the tissue damage normally characteristic of the chronic phase of infection [66]. Although these data validate genetic vaccination as a means to control *T. cruzi* induced mortality, a genetic vaccine candidate(s) has still not been identified that can protect 100% of animals from lethal infection nor protect against subsequent development of chronic disease. In addition, an effective field vaccine will have to protect a diverse human population from infection with a diverse parasite population and thus will probably require a complex mixture of genes. Therefore there continues to be a

need for identification and testing of additional vaccine candidates. Background on the vaccine candidates tested in the study detailed in Chapter two is included below.

LYT1. LYT1 was identified from a cDNA library using antibodies against the C9 component of the membrane attack complex of complement [71]. It had been demonstrated that antibodies against C9 recognize a secreted acid-stable protein from *T. cruzi*, TC-TOX, that has pore-forming activity at low pH and is thought to be involved in the escape of the parasite from the parasitophorous vacuole [6-8]. The gene for TC-TOX has not been cloned but the fact that both TC-TOX and LYT1 have hemolytic activity, are similar in size and react with antibodies against C9 suggest that, if not one in the same, these two proteins are at least functionally related. LYT-1 deficient parasites have decreased infectivity, decreased hemolysis and enhanced development [71].

Flagellar Ca^{2+} binding protein, FCaBP. A 24-kDa calcium binding protein that primarily localizes to the flagellum [72] and is also associated with the cell surface and with vesicles released from the parasite [73] has been repeatedly identified from *T. cruzi* cDNA expression libraries (Chapter two)[73-75]. The gene encoding FCaBP is tandemly repeated and the resultant mRNA composes 5% of the total poly $(A)^+$ RNA in the cell [74]. This protein has four putative EF-hand domains and binds calcium through the third and fourth [76]. The flagellar localization is mediated by N-terminal myristoylation and palmitoylation and further regulated by the binding of calcium, making it a calcium-myristoyl/plamitoyl switch protein [72]. Although the specific function of FCaBP has not been characterized, it is thought to play a role in the rapid motility of the trypanosomes [75]. Immunization with recombinant FCaBP induced partial protection against a lethal challenge of *T. cruzi* [77].

Trans-sialidases. The *T. cruzi* trans-sialidase (TS) gene superfamily is estimated to be composed of more than 600 genes [68, 69], the majority of which do not encode enzymatically active trans-sialidase molecules. The TS gene superfamily can be divided into two subfamilies, the TS family and the TS-like family [69]. The TS family is composed of approximately 140 members, half of which are capable of transferring sialic acid from host sialoglycoconjugates to β-galactoses in the glycoconjugates of the parasite [70]. This activity is significant because *T. cruzi* is not capable of synthesizing sialic acid *de novo.* The TS-like family consists of hundreds of members that share 30-40% amino acid identity to the TS family members however they are all enzymatically inactive. These molecules have been shown to be involved in cell invasion and resistance to complement (reviewed in [70]).

Mucins. It is estimated that the mucin gene family of *T. cruzi* is composed of over 700 genes [68]. These genes are highly expressed and encode highly glycosylated GPI-anchored surface proteins that are the major acceptors of sialic acid on the parasite surface [78]. Mucins are shed from the parasite surface and have been shown to be important for host-parasite interactions and protection from complement mediated lysis (reviewed in [69]). Together the mucin and TS super gene family members make up 5% of the parasite genome and are the most abundant proteins on the parasite surface.

Adaptins and vesicle trafficking

Adaptor protein complexes are integral components of vesicle-mediated protein trafficking in the endocytic and secretory pathways, recruiting and specifying both the coat protein and the vesicles' cargo [79]. To date four adaptor protein complexes have been identified, AP-1- AP-4 [79-82] (AP-3 is illustrated in Figure 1.1). All of the adaptor

complexes are heterotetramers consisting of two large subunits (γ/α/δ/ε and β1-β4 ~90-130 kDa) one medium subunit (μ1-μ4 ~50 kDa) and one smaller subunit σ1-σ4 ~20 kDa) [82, 83].

One of the best-characterized vesicle coats is composed of adaptor protein complexes (APs) and clathrin (reviewed in [84]). Clathrin coated vesicles bud from two distinct areas in the cell, the plasma membrane and the trans-Golgi network (TGN). The clathrin-coated vesicles originating at the plasma membrane contain the AP-2 adaptor protein complex whereas AP-1 is associated with clathrin-coated vesicles originating from the TGN. One of the initial steps in clathrin coat formation is the recruitment of APs from the cytosol to the membrane where they bind the cytoplasmic domains of cargo proteins, usually transmembrane receptors.

Although the factors responsible for this recruitment are still being identified, it is known that the μ subunits of the APs interact with tyrosine-based sorting signals within the cytoplasmic domains of cargo proteins [85, 86]. In addition, adaptor complexes recognize di-leucine based sorting signals in the cytoplasmic tails of target proteins mediated via the β subunit [87-89]. Signals in the cytoplasmic tails are not thought to be the only factors attracting the APs to the membrane. It has been proposed that membrane-bound docking proteins specific for the various APs exist but their identity is still largely unknown [90, 91].

Interaction between the AP-1 and AP-2 complexes with clathrin is mediated by the respective AP β subunits [92-95]. Initial characterization of AP-3 coated vesicles revealed that their coat was much thinner than a typical clathrin coat indicating that unlike AP-1 and AP-2, the AP-3 complex may not associate with clathrin. The findings

that AP-3 subunits are not detectable by Western blot analysis of preparations from clathrin-coated vesicles and that in vitro AP-3 driven vesiculation is not clathrin-dependent bolstered this observation [96, 97]. Although the majority of literature is in agreement that these vesicles are not clathrin-coated, there is one report demonstrating that the AP-3 β subunit is capable of interacting with clathrin in vitro and co-localization of AP-3 and clathrin on intracellular vesicles [98]. Therefore there is still a degree of controversy concerning the identity of the coat protein associated with these vesicles.

Two isoforms of the AP-3 β subunit, β3A and β3B, have been identified in mice and humans. β3B was the first β subunit of the AP-3 complex to be identified and was identified during an attempt to find neuron-specific proteins by screening a human cerebellar cDNA expression library with antiserum from an individual suffering from cerebellar degeneration [98]. This 120 kDa protein is 30% identical to the β adaptins of the AP-1 and AP-2 complexes and initially named β-NAP (for neuronal adaptin-like protein). Localization studies revealed that β-NAP is associated with vesicles at the nerve terminus as well as being found free and membrane-bound in the cytoplasm of the cell body and the axon terminus [96]. Further investigation revealed that β-NAP is a part of a unique heterotetrameric complex subsequently named AP-3. Two of the subunits, β-NAP and μ3B, are only expressed in neurons but ubiquitously expressed isoforms have now been identified and are referred to as β3A and μ3A [80, 81, 99]. The amino acid sequences of β-NAP and β3A are 61% identical and 75% similar to one another [100]. Following the identification of β3A, β-NAP was renamed β3B.

Based on its localization, the neuro-specific AP-3 complex was originally postulated to direct sorting and trafficking of vesicle membrane proteins between the cell

body and the axonal terminus in neurons [96]. It has now been well established that one function of the AP-3 complex is synaptic vesicle biogenesis [97, 101, 102]. Synaptic vesicles (SVs) are small vesicles found at the nerve terminus that house neurotransmitters (reviewed in [103]). Upon excitation, these vesicles rapidly fuse with the plasma membrane in a calcium-dependant manner releasing their contents into the synapse [104]. This event can be triggered up to and possibly surpassing 1000 times per second. Therefore these vesicles must be rapidly regenerated via endocytosis from the plasma membrane and a very rapid rate of endocytosis at the axonal terminus has been documented [105]. The reconstitution of these vesicles also requires an intricate sorting process where specific cargo is reproducibly recruited while resident proteins of the donor membrane are excluded. The mechanisms responsible for this process are beginning to be elucidated. It is now known that the majority of SVs originate directly from the plasma membrane in an AP-2/ clathrin-dependent manner however a subset is formed from endosomes in an AP-3 dependent/ clathrin-independent manner [97]. It has been demonstrated that AP-3 association with transferrin (Tf) containing endosomes results in sorting of specific membrane proteins and subsequent budding of vesicles that are devoid of Tf but enriched in SV specific proteins like VAMP-2, a v-SNARE that is essential for fusion of the SV to the plasma membrane and may also play a role in the recruitment of the AP-3 complex [104, 106].

Genetic studies in yeast, Drosophila and mice have further elucidated the functions of the AP-3 complex and indicate that it is involved in protein sorting and vesiculation from the TGN and/or endosomes and the delivery of these proteins to lysosomes and lysosomal-like compartments including pigment and platelet-dense

granules [107-110]. Although there is not a complete understanding of how the neuro-specific AP-3 complex fits into this picture, characterization of two mouse mutants, the *mocha* and *pearl* mice, has confirmed the neuro-specific role of AP-3. *Pearl* mice express an extremely low level of the β3A subunit of the AP-3 complex, the ubiquitously expressed β isoform, and the expressed protein is severely truncated. These mice suffer from hypopigmentation, lysosomal secretion abnormalities and prolonged bleeding due to storage pool deficiency in the platelet-dense granules [111]. In addition to these symptoms, *mocha* mice, which fail to express the δ subunit of the AP-3 complex, suffer from neurological defects including hyperactivity, EEG abnormalities, seizures and deafness [112-114]. Even though both strains of mice are each lacking one subunit of the AP-3 complex only one strain exhibits neurological defects. This can be explained by the fact that the pearl mutant, which lacks the ubiquitously expressed β3A subunit, still expresses normal levels of the neuro-specific β3B whereas the mocha mice lack the δ subunit of which there are no variant isoforms [115]. The pearl mice are capable of forming functional neuronal AP-3 complexes utilizing the β3B subunit, but they cannot construct the AP-3 complex in other cell types due to the lack of the β3A subunit and consequently they exhibit the phenotypes associated with the loss of function of the ubiquitously expressed AP-3 complex. In contrast, mocha mice are not capable of assembling either the neuronal or non-neuronal AP-3 complexes, which is illustrated by the additional neurological defects that they exhibit. Further characterization of these neuronal defects revealed that there was a drastic reduction in zinc in normally zinc-rich neurotransmitter vesicles throughout the neurons in the central nervous system and that this was correlated to the mislocalization of a neuron specific zinc transporter [109].

These results suggest that the neuronal AP-3 complex is required for the accurate sorting of this particular zinc transporter and may indicate that zinc concentration is related to the neuronal defects seen in these animals though these defects could also be caused by the misrouting of other proteins that has not yet been detected. Despite the deficiency in the formation of zinc-containing synaptic vesicles other synaptic vesicles were not affected, reinforcing the in vitro finding that two distinct routes can lead to the formation of synaptic vesicles, only one dependant on AP-3, and also suggesting that AP-3 may be required for the formation of a specialized subset of synaptic vesicles [97, 101, 109].

The phenotypes seen in the *pearl* and *mocha* mice resemble the symptoms exhibited in patients suffering from Hermansky-pudlak syndrome (HPS) [116]. HPS is characterized by abnormalities in lysosomal-like organelles like melanosomes and platelet-dense granules. Although mutations in various genetic loci can lead to HPS [117], it has just recently been demonstrated that a subset of HPS patients has mutations in the β3A subunit of the AP-3 complex [118]. Fibroblast cells from these patients have dramatically reduced levels of the AP-3 complex accompanied by increased surface expression of several lysosomal membrane proteins [118]. These experiments indicate that mutations in the β3 subunit may be related to the symptoms manifested in these HPS patients.

Although it has been clearly demonstrated that neuronal AP-3 can function to make synaptic vesicles from endosomes, that the absence of neuronal AP-3 causes neurological abnormalities, and that the ubiquitously expressed AP-3 complex directs trafficking of specific proteins to lysosomal like compartments, many questions remain [88, 102, 107, 109, 110]. The mechanisms underlying the different functions of the

neuro-specific and ubiquitous AP-3 complexes are not fully understood and delineating the roles of these similar complexes may be difficult in cells that express both the ubiquitous and the neuro-specific subunits. Recent evidence from Drosophila indicates that there is only one β3 subunit in this organism that is equally identical to human β3A and β3B, 51% and 54% respectively, and much less similar to the β subunits of the other adaptor complexes [119]. Mutations in this gene lead to lysosomal-like organelle defects however there are conflicting reports concerning neuronal/behavioral defects in AP-3 mutant Drosophila [119, 120]. If confirmed, the involvement of Drosophila's β3 adaptin in both neuronal and lysosomal trafficking might suggest that an evolutionary precursor of β3A and β3B may have been capable of functioning in a more general capacity.

Vesicle trafficking in trypanosomes

There is an extremely limited understanding of protein trafficking in *T. cruzi* as well as other kinetoplastids like *Trypanosoma brucei* and *Leishmania* the causative agents of Chagas disease, African sleeping sickness and leishmaniasis respectively. Although receptor-mediated endocytosis and the existence of coated vesicles have been documented in these organisms the adaptor and coat proteins involved in these processes have not been functionally characterized [121-127]. Unique structural constraints limit endo- and exocytosis to a small portion of the surface membrane. Trypanosomes are highly polarized cells that have three very distinct surface membrane domains, the plasma membrane, the flagellar membrane and the membrane of the flagellar pocket. Even though the three domains are all a part of a continuous lipid bilayer, they each have very distinct characteristics and functions maintained by differential protein composition.

The membrane of the flagellar pocket is particularly interesting because all of endocytosis and secretion in these organisms is restricted to this area of the surface membrane (reviewed in [128]). This restriction is related to the presence of a subpellicular corset of microtubules that blocks interaction and fusion of cytoplasmic vesicles with the plasma membrane. The subpellicular cortex is composed of more than 100 closely spaced microtubules that lie directly beneath the plasma membrane. However these microtubules are not present underneath the membrane of the flagellar pocket therefore not restricting trafficking at the flagellar pocket membrane domain [128, 129]. The flagellar pocket is formed by an invagination of the plasma membrane in the area where the flagellum emerges from the cell body [130]. There is an extremely rapid rate of membrane turnover in the flagellar pocket membrane [128], which coincides with the demands placed on this membrane due to its role as the sole location of import and export in the entire parasite. The rapid rate of endocytosis and membrane turnover at the flagellar pocket is somewhat reminiscent of that at the axonal terminus [105]. The neuro-specific AP-3 complex has been shown to be critical for a synaptic vesicle biogenesis and seems to play a role in vesicular trafficking from the cell body to the axonal terminus [131].

It has been documented that macromolecules, like LDL and transferrin, are internalized at the flagellar pocket membrane, via receptor-mediated endocytosis, associate with vesicles that are scattered from the flagellar pocket region to the posterior end of the cell eventually culminating in acidic reservosomes [124, 132]. These reservosomes are similar to lysosomes, are sites of accumulation of ingested proteins and lipids, account for 5-6% of the cell volume, and may be critical for life-cycle stage

transformation [133]. In higher eukaryotic cells the ubiquitous AP-3 complex has been shown to be involved in vesicular trafficking to lysosomes and lysosomal-like compartments.

The fortuitous cloning of an apparent β3 adaptin in *T. cruzi* and subsequently in *T. brucei* provided an opportunity to explore the localization and function of β3 adaptins in trypanosomes (Chapter 3).

RNA interference

Double-stranded RNA molecules have been demonstrated to mediate post-transcriptional gene silencing (PTGS), a system that is thought to have evolved as a defense mechanism against viruses before the divergence of plants and animals [134-136]. It was initially shown that PTGS can be induced by the introduction of dsRNA in *Caenorhabditis elegans* [137] and has now been shown to be effective in many different organisms including *Trypanosoma brucei* (initially described in [138]). The specific mechanism of RNAi is still being elucidated but the current models suggest that dsRNA molecules are processed into 21-23 nt RNAs termed short interfering RNAs (siRNAs) (reviewed in [135]. These siRNAs direct the sequence specific degradation of corresponding mRNA molecules.

RNAi of various genes in *T. brucei* has led to distinct phenotypes. For example, RNAi of α-tubulin leads to the "FAT" phenotype, parasites lose their slender morphology and become multinucleate, indicating a block in cytokinesis [138] and RNAi of FLA1 in *T. brucei*, a homologue of a 73 kDa protein from *T. cruzi* that plays a role in flagellar adhesion [139], results in parasites with detached flagella [140]. In addition RNAi can be

regulated in *T. brucei* by transfecting cells that express both the T7 polymerase and the tetracycline repressor [141] with constructs containing a fragment of the gene of interest flanked by dual tetracycline regulated T7 promoters. Generation of the dsRNA is induced by the addition of tetracycline. The ability to regulate the production of dsRNA allows the analysis of potentially lethal dsRNAs.

RNAi has yet to be definitively demonstrated in *T. cruzi* and more importantly a widely usable regulated expression system has not been developed in *T. cruzi*, thus hampering the ability to use RNAi to analyze the role of essential genes. Therefore we developed a plasmid that would allow the generation of dsRNA and impart the ability to detect and purify transfectants shortly after transfection by fluorescence based cell sorting. This was achieved by replacing the drug resistance marker of a dsRNA generating plasmid that has been extensively used in *T. brucei*, pZJM [142], with a chimeric gene encoding both GFP and puromycin resistance, EGFP-puro [143]. This plasmid was capable of conferring GFP expression, puromycin resistance and dsRNA induced phenotypes in *T. brucei*, we are using it to investigate RNAi in *T. cruzi* and we believe will be a powerful tool for analyzing the effects of dsRNA from potentially lethal genes in organisms where regulated expression systems have not been developed.

As RNAi is still being worked out in *T. cruzi*, we used the powerful regulated inducible RNAi system in *T. brucei* to assess the function of a *T. cruzi* gene by using its sequence to find the *T. brucei* homologue and analyzing the effects of RNAi of this gene in *T. brucei* (Chapter 3).

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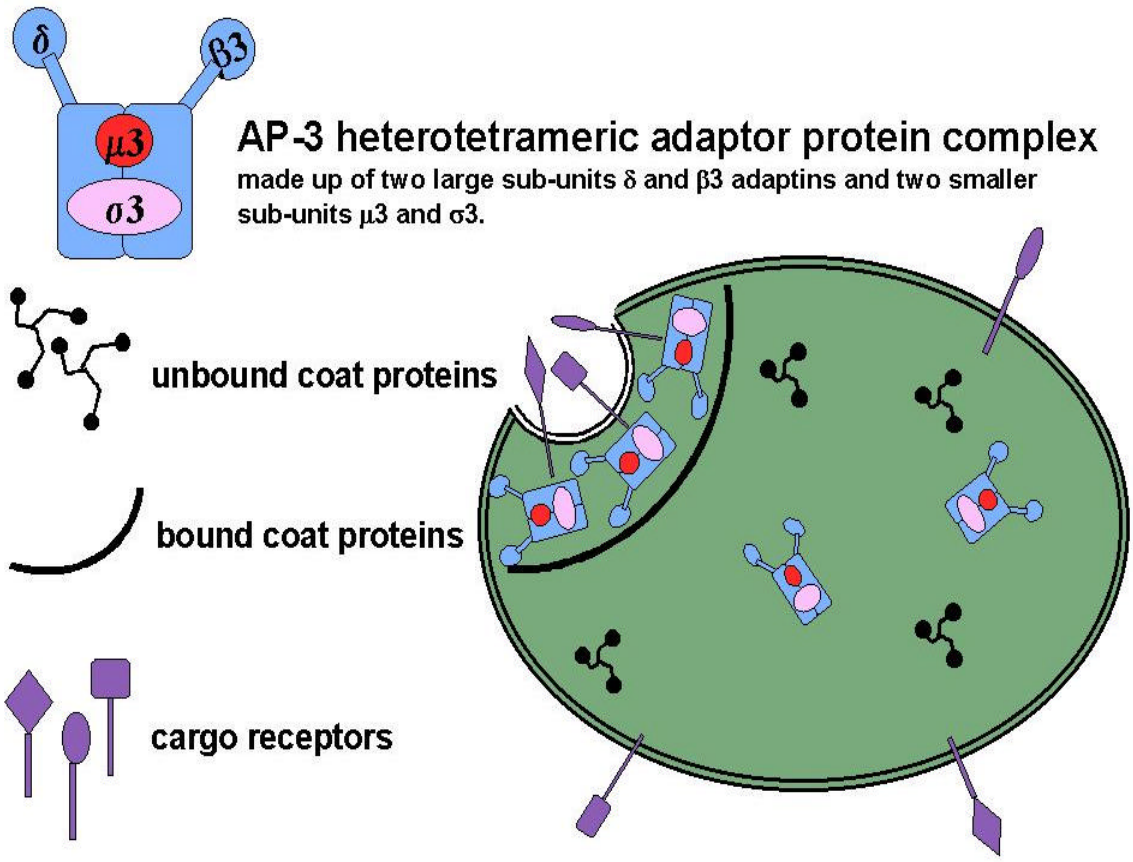

Figure 1.1. Illustration of the AP-3 heterotetrameric adaptor protein complex (APs) and a more generalized illustration of the recruitment of APs from the cytosol to the membrane where they bind the cytoplasmic domains of cargo proteins, usually transmembrane receptors, in addition to binding coat proteins.

CHAPTER 2

GENETIC IMMUNIZATION WITH LYT1 OR POOL OF TRANS-SIALIDASE GENES PROTECTS MICE FROM LETHAL *TRYPANOSOMA CRUZI* INFECTION[1]

[1] Fralish, B. H. and R. L. Tarleton. To be submitted to *Vaccine*.

Abstract

Genetic immunization has been demonstrated to be an effective means of protecting mice from a normally lethal challenge of *Trypanosoma cruzi*. Although a number of parasite molecules have been tested as genetic vaccines for *T. cruzi*, the majority are members of the trans-sialidase (TS) gene superfamily. The goal of this study was to expand the diversity of genes assessed as genetic vaccine candidates both outside and within the TS gene family. Genes outside of the TS gene family were identified by screening a *T. cruzi* amastigote cDNA expression library with anti-amastigote monoclonal antibodies, resulting in the identification of two genes, the previously identified Ca^{2+} binding protein, *FCaBP*, and a novel homologue of the adaptin AP-3 complex β3 subunit, *Tcb3*. A third gene, *LYT1*, was recently identified as a secreted *T. cruzi* protein involved in cell lysis and infectivity, and was selected because of its expression and secretion from the intracellular stage of the parasite. Although peptides from all three genes were found to be targets of cytotoxic T cell responses in chronically infected mice, only immunization with *LYT1* protected mice from a normally lethal challenge of *T. cruzi.* As an alternative to testing individual *T. cruzi* genes as vaccines, pools of genes from the TS and mucin families were assessed in vaccination studies. The mucin family was selected because like the TS family it encodes GPI-anchored surface proteins that are abundantly expressed. Immunization with pools of TS but not mucin genes provided protection against a normally lethal challenge of *T. cruzi.* This study demonstrates that the ability of *T. cruzi* proteins to elicit immune responses in infected hosts does not necessarily associate with the ability to induce protection and that both the products of single genes and multi-gene families may serve as effective vaccines.

Furthermore these results establish the approach of using pools of multi-gene family members as an effective option for vaccination in *T. cruzi* infection.

Introduction

Fifty thousand people die each year due to infection with the protozoan parasite *Trypanosoma cruzi*. A total of 16-18 million people are currently infected and another 100 million are at risk of infection throughout Central and South America [1, 2]. Infection in humans normally progresses from an acute stage characterized by systemic parasitemia to a virtually asymptomatic stage that can last for several decades. Eventually, approximately one third of those chronically infected begin to manifest the symptoms of Chagas disease, developing severe cardiac and/or gastrointestinal disease, which in most cases leads to death. There is no cure for Chagas disease but two drugs, nifurtimox and benznidazole, can be effective to treat infection with *T. cruzi*. Unfortunately the availability and use of these drugs is limited due to their extreme toxicity. Chagas disease is the most serious parasitic disease in Latin America and is amongst its leading health problems.

The severity of this problem, the lack of adequate chemotherapeutics and the increasing evidence connecting parasite persistence and disease development (reviewed in [3]) justifies pursuing vaccine development for *T. cruzi* infection.

Genetic vaccination has been shown to generate both MHC class I- and MHC class II-restricted T cell responses leading to the activation of $CD8^+$ and $CD4^+$ T cell populations (reviewed in [4]), which are critical for control of *T. cruzi* infection [5, 6]. A number of studies have demonstrated the potential of genetic immunization as a means to protect against *T. cruzi* infection [7-12]. Most of these studies focus on members of the trans-sialidase gene family encoding glycosylphosphatidylinositol (GPI)-anchored surface proteins many of which are not enzymatically active (reviewed in [13]). Vaccination

with various TS family members provided significant protection against *T. cruzi* induced mortality and has been shown to significantly reduce the tissue damage normally characteristic of the chronic phase of infection [12].

Although vaccination with the TS genes provides significant control of the infection, vaccination with the individual TS genes alone or in combination failed to inhibit the establishment of infection, induce parasite clearance or protect 100% of the mice. In order to address whether or not the partial protection provided by genetic vaccination with the TS family members could be improved by vaccinating with genes outside of this family, alone or in addition to the TS genes, additional vaccine candidates must be identified and evaluated. This study tested three non-trans-sialidase genes and found that one, *LYT1* [14], induced a protective response against *T. cruzi* infection in a murine model. The level of protection generated by vaccinating with *LYT1* was then compared to that generated by vaccinating with a pool of TS genes that had not been previously tested as well as a pool of mucin genes, another large gene family that also encodes GPI-anchored surface proteins (reviewed in [15]). We found that immunization with LYT1 or the trans-sialidase pool, but not the mucin pool, induced protection in mice.

Materials and Methods

Parasites and mice. The Brazil strain of *T. cruzi* was used in these experiments and was maintained in vivo by serial biweekly passage of 10^3 blood-form trypomastigotes (BFT) in C3H/HeSnJ mice [16]. Parasites were maintained in vitro via passage of tissue culture-derived trypomastigotes in monolayers of Vero cells (African green monkey

kidney cells) [17]. C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) female mice, six to eight weeks of age, were used in this study.

Cell lines and culture reagents. RMA-S cells (an immuno-selected variant of the RBL-5 lymphoma that are peptide TAP-2 transporter-deficient and therefore deficient in the expression of class I MHC molecules, kindly provided by Dr. M.B. Oldstone, Scripps Research Institute, La Jolla, CA) were cultured in complete RPMI-1640 medium (CM; Mediatech, Herndon, VA) containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 50 μg/ml gentamicin (all from Gibco BRL, Gaithersburg, MD). Vero cells (African green monkey kidney cells) and COS-7 cells (simian virus 40-transformed African Green monkey kidney cells) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and were cultured in CM and Dulbecco's modified Eagle's medium (DMEM, supplemented as just described; Mediatech, Herndon, VA) respectively. T cell medium (TCM) was prepared by supplementing CM with 0.1 mM non-essential amino acids (Gibco, BRL).

cDNA library screening. *T. cruzi* specific monoclonal antibodies, generated as described [18], were used to screen a previously constructed *T. cruzi* amastigote cDNA expression library (cloned into Uni-ZAP XR expression vector, Stratagene, La Jolla, CA) also described in [18]. Fifty plates (150 mm) containing a total of 10^5 plaque-forming units were screened with various monoclonal antibodies. Positive plaques were identified by two different monoclonal antibodies, IIID4 and VIIA4. Three rounds of screening

were performed for each positive plaque before in vivo excision of the pBluescript II (SK-) containing insert from Uni-Zap XR according to the manufacturer's instructions (Stratagene, La Jolla, CA).

Western blots. *T. cruzi* amastigotes were harvested from Vero cell cultures and resuspended at 10^9 parasites/ml in lysis buffer (10 mM Tris-HCL, pH 7.5; 5mM EDTA; 1% Nonidet P-40; 1 mM phenyl-methanesulfonyl fluouride; 10 mg/ml aprotinin; 50 U/l trasylol; 10 mg/l leupeptin) by repeated freeze-thaw treatment. The lysates were cleared by centrifugation (30 minutes, 4°C at 14,000 x *g*), supernatants collected and separated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) by semi-dry electroblotting for 2h at 20 V at room temperature in Bjerrum and Schafer-Niesen buffer (48 mM Tris base; 39 mM Glycine; 200 ml/l methanol; 0.04% SDS)[19]. Membranes were blocked in PBS containing 0.5% non-fat dry milk, 0.1% Tween-20, incubated with respective monoclonal antibodies for 1 h at room temperature, and then with a horseradish peroxidase (HRP) labeled goat anti-mouse IgG and IgM (Accurate Chemical and Scientific Corp., Westbury, NY), 1 h at room temperature. Blots were developed with 1.6 mM 3,3' diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M Tris-HCL pH 7.6 containing 0.03% H_2O_2 at room temperature until stopped with H_2O.

Flow cytometric analysis. Amastigotes harvested from infected Vero cells were washed in PAB (0.1% BSA, 0.1% sodium azide in PBS), resuspended at 10^6/ 50 µl in PAB containing the mAb culture supernatants (diluted 1:1) and incubated for 30 minutes at

4°C. OKT4, a monoclonal antibody that recognizes human helper T-cells (ATCC-CRL 8002), was used as a negative control. The cells were then washed in PAB, stained for 30 minutes at 4°C with fluorescein isothiocyanate (FITC)-labeled goat $F(ab')_2$ anti-mouse IgG (Pharmingen, San Diego, CA; diluted 1:50 in PAB) washed again in PAB and then analyzed by flow cytometry on an Coulter Elite Analyzer (Beckman Coulter, Inc., Fullerton, CA).

DNA sequencing. The cDNA clones were sequenced by the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia, using the Taq dye terminator chemistry on an ABI 373 DNA sequencer (Foster City, CA). Sequence homology searches of the database maintained at the National Center for Biotechnology Information (NCBI; Bethesda, MD) were carried out using the BLAST program.

Peptides. The sequences for the *T. cruzi* proteins FCaBP, Lyt-1 and Tcβ3 were scanned for peptides that match the murine H2-K^b allele-specific class I peptide binding motif using the HLA Peptide Binding Predictions program from BioInformatics & Molecular Analysis Section (BIMAS), http://bimas.dcrt.nih.gov/molbio/hla_bind. Several peptides from each protein predicted to bind to H-2K^b were synthesized using Fmoc-based solid phase chemistry on an ACT MPS 350 peptide synthesizer (Advanced Chem Tech, Louisville, KY) by MGIF at the University of Georgia. Known H-2K^b restricted CTL epitope's from *T. cruzi's* trans-sialidase protein ASP-1, PA14 ($ASP\text{-}1_{509\text{-}516}$) [20] and from chicken ovalbumin, $OVA_{257\text{-}264}$ (SIINFEKL), were used as control peptides. Lyophilized peptides were dissolved at 20 mg/ml in dimethyl sulfoxide and stored at

-70°C. Peptides were diluted in RPMI 1640 before use and were not toxic to target or effector cell cultures. Peptides reported in this study derived from Tcβ3, FCaBP and LYT1 are; Tcβ3 p1 (SLFGYRKL), FCaBP p4 (PAALFKEL) and LYT1 p5 (ELTMYKQLL).

Cytotoxic T cell assay. Effector cells were generated by culturing 5 x 10^6 spleen cells from chronically infected mice (2.5 x 10^6/ml TCM 2 ml/well in 24 well plates) with 1μM peptide at 37°C in 5% CO_2. After 48 hours the culture media was supplemented with 5% Rat T-STIM without Con A (Collaborative Biomedical Products, Bedford, MA) and incubated for 4 more days. RMA-S (H-2K^b) target cells were incubated at 26°C for 24 hours and then plated 10^6 cells/2 ml/ well in 24 well plates with 1 μM peptide and 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, IL) overnight at 26°C, 5% CO_2. The target cells were incubated at 37°C before being processed for CTL assay as described [20, 21]. Briefly, ^{51}Cr-labeled target cells were washed 3 times and resuspended in TCM, 5 x 10^3 were added to effector cells at various effector/target ratios in 96-well round – bottom plates (Corning). After a 5 hour incubation at 37°C, the supernatants were harvested with the SCS system (Skatron, Sterling, VA) and radioactivity was counted on a Cobra II Autogamma counter (Packard Instrument Company, Downers Grove, IL). Percent specific lysis was determined from the mean of triplicates as 100 X [(experimental release – spontaneous release)/(maximum release - spontaneous release)]. Maximum and spontaneous release were defined by the counts from target cells incubated with and without 2% Triton X-100, respectively.

Plasmid construction. FCaBP was PCR amplified from pBluescript II (SK-) with a gene specific primer that incorporated a Bgl II site (underlined), 5'-GAAGATCTGGGTGCTTGTGGGTCGAA-3' and T7 primer 5'-TAATACGACTCACTATAGGG-3', this PCR product was cloned into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) and subsequently released by digestion with Bgl II and EcoRV and inserted into pCMVI.UBF3/2 (pCMV; provided by Drs. Kathryn Sykes and Stephan A. Johnston, University of Texas Southwestern Medical Center, TX) that had been digested with Bgl II and Sma I. The first 403 bp of Tcβ3 was PCR amplified from pBluescript II (SK-) containing Tcβ3 with gene specific forward primer that incorporates a Bgl II site (underlined), 5'-GAAGATCTGAAGGCTACATTGGCGTC-3' and a gene specific reverse primer that spans a Sph I site (underlined), 5'-GCGTACATGCATGCTTGGGTCCA-3'. The remainder of Tcβ3 was released from pBluescript II (SK-) containing Tcβ3 by digesting with Sph I (internal to Tcβ3) and Xho I (3' of TcB3 in the multiple cloning site of pBluescript II (SK-). These fragments were ligated to each other and then into pCMV that had been digested with Bgl II and Xho I. *T. cruzi's* LYT1 gene (GenBank Accession Number; AF320626) was PCR amplified from pBSLYT1 (generously donated by Dr. John Swindle, Infection Disease Research Institute, Seattle, WA) with gene specific forward and reverse primers introducing BamH I and Sal I sites respectively (sites underlined), 5'-CGGGATCCAGAAAGCCGCAGCATTA-3' and 5'-ACGCGTCGACTTCAATCAGCTGCCAGCA-3'. The PCR amplified product was ligated into pCMV that had been digested with BamH I and Sal I. The resulting

constructs, pCMV.*FCaBP*, pCMV.*Tcb3* and pCMV.*LYT1*, were confirmed by DNA sequencing at the MGIF (University of Georgia, Athens, GA) as described above.

Multiple sequence alignments of selected genes from the trans-sialidase and mucin gene families were used to design primers, based on conserved 5' and 3' sequences, for PCR amplification and directional cloning into the pCMV vector. PCR primers were based on the following trans-sialidase genes listed by Genbank accession numbers; D50684, D50685, D50686, AJ276679, U01098, U50162, L26499, L38456, L38457, L38463. The forward and reverse primers introduced an EcoR V and Xba I sites respectively (sites underlined), 5'-CGATGATATCCAAAGTCACCGAGCGGGTTG-3' and 5'-GCGCTCTAGACGTCTGGCACAACGGTCTG-3'. PCR primers designed to amplify mucin genes were based on the following mucin genes listed by Genbank accession numbers; AF036441, AF036443, AF036445, AF036450, AF036451, AF036454, AF036456, AF036462, AF036463, AF036464, AF036465, AY032683. The forward and reverse primers introduced BamH I and Xba I sites respectively (sites underlined), 5'-CGGGATCCYTGTGCTGCTGCCCGTCCGT-3' and 5'-GCTCTAGATTAGGTGTACGCCAGCGCGAT-3'. These primers were used to PCR amplify genes from cDNA prepared from amastigote RNA. Briefly, RNA was isolated from 5 x 10^8 culture derived amastigotes using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to manufacturers instructions. 5 µg of amastigote RNA was used for cDNA synthesis with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) following manufacturers guidelines. The PCR amplified products were gel purified with QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) and then the ts

genes were digested with EcoR V and Xba I and the mucin genes with BamH I and Xba I. The digested products were purified using YM-100 Microcon Centrifugal Filter Devices (Millipore, Corp., Bedford, MA), washing 3 times to remove fragments smaller than 125bp. The trans-sialidase PCR amplified digested products were then ligated into pCMV that had been digested with BamH I, treated with T4 DNA Polymerase (Promega, Madison, WI) to create a blunt end and then digested with Xba I. The mucin PCR amplified digested products were ligated into pCMV that had been digested with BamH I and Xba I. Several resultant colonies were sequenced, with a primer that hybridizes 5' to the insertion site 5'-CCCTGACCGGCAAGACCA-3', to confirm that trans-sialidase and mucin sequences had been inserted into pCMV, sequences were compared to databases maintained NCBI using the BLAST program.

Plasmids encoding murine IL-12 and GM-CSF (pcDNA3.msp35, pcDNA3.msp40 and pCMVI.GM-CSF) were provided by Dr. S.A. Johnston.

Plasmids were transformed into *E. coli* DH5-alpha competent cells and grown in Luria broth (LB) supplemented with 100 µg/ ml ampicillin. DNA was purified by using the QIAGEN EndoFree Plasmid Mega Kit (QIAGEN) according to manufacturer's instructions. Approximately 150 clones from the trans-sialidase and mucin transformations were pooled and resuspended in 2.5 mls of LB, 500 µl of this was used to inoculate 500 mls of LB from which DNA was purified.

Oligonucleotides were constructed on an Applied Biosystems 394 DNA/RNA synthesizer (Foster City, CA) at MGIF.

Heterologous expression. Expression of gene products cloned into pCMVI.UBF3/2 (pCMV) was confirmed by transient transfection of COS-7 cells with pCMVI.UBF3/2.*Tcb3* (pCMV.*TcB3*) and pCMVI.UBF3/2.*FCaBP* (pCMV.*FCaBP*) as described [22]. Briefly, 10^5 COS-7 cells plated in 6-well plates were transfected with 5 µg of DNA (each plasmid separately) using Lipofectin (Gibco BRL, location?). 48 hours later cells were transferred to 8-well Lab Tek chamber slides (Nunc Inc., Naperville, IL) at 10^4 cells/well and incubated for 24 hours. The cells were then fixed with ice cold methanol, blocked with 1% bovine serum albumin (BSA) in PBS, and stained with mouse monoclonal antibodies specific for FCaBP or TcB3, IIID4 and VIIA4 respectively, diluted 1:200 in PBS-1% BSA. Cells were then stained with FITC-labeled $F(ab')_2$ goat anti-mouse IgG diluted 1:50 in PBS-1% BSA (Southern Biotech., Birmingham, AL)[22]. Slides were mounted in 10% glycerol, 0.1 M sodium bicarbonate (pH 9), 2.5% 1,4-diazobicyclo-(2,2,2) octane, and visualized by laser scanning confocal microscopy (MRC-600, Bio-Rad Laboratories, Hercules, CA).

Genetic immunization. Endofree plasmid DNA (25 µg, unless otherwise noted) was injected into the quadricep muscle of 6-8 week old female C57BL/6J mice and these mice were boosted with the same dose six weeks later. Two weeks after the boost the mice were infected via intra-peritoneal injection of a normally lethal dose of the Brazil strain of *T. cruzi* (1-2.5 x 10^5 blood form trypomastigotes/mouse). In some experiments mice were also immunized with cytokine encoding plasmids (25 µg each, pcDNA3.msp35, pcDNA3.msp40 and pCMVI.GM-CSF). Mortality was recorded daily.

Interferon-gamma ELISPOT assays. The enzyme-linked immunospot (ELISPOT) assay was used to quantitate peptide specific interferon gamma (IFN-γ) producing cells [23]. Nitrocellulose bottom 96 well plates (Millipore, Corp., Bedford, MA) were coated with anti-mouse IFN-γ Ab (R4-6A2, 10 μg/ml in PBS) overnight at 4°C, washed 3 times with PBS and then blocked with 100 μl/well complete-RPMI for 1 hour at room temperature. Spleen cells from immunized mice were collected 2 weeks after the final immunization and plated at 4 x 10^5 cells/well (100 μl/well) in triplicate with or without 10 μg/ml peptide (Tcβ3 p1, FCaBP p4, and LYT1 p5 respectively) and incubated at 37°C, 5% CO_2 overnight. Plates were washed 8 times with PBS containing 0.05% Tween (PBS-T) and incubated with 100 μl polyclonal rabbit anti-mouse IFN-γ [24] diluted 1:1000 in PBS-T overnight at 4°C. Plates were washed 8 times in PBS-T and incubated with 100 μl peroxidase-labeled goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in PBS-T. Again plates were washed (8 times in PBS-T and 2 times PBS) and spots were developed by adding 100 μl of freshly prepared developing solution (5 mg of 3-amino-9-ethylcarbazole dissolved in 500 μl of dimethylformamide was added to 14.5 mls of 0.1 M sodium acetate buffer pH 4.8, the solution was filtered and 7.5 μl of H_2O_2 was added.). Spots developed within 5-10 minutes, plates were washed with dH_2O, air-dried and spots were counted using a dissecting microscope.

Histology. Mice were sacrificed and skeletal muscle was removed, fixed in 10% buffered formalin for 48 hours, dehydrated in absolute ethanol, cleared in xylene and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin and evaluated by light microscopy.

Results

Gene identification. In an attempt to identify genes from *T. cruzi* for evaluation as genetic vaccines, an amastigote cDNA expression library was screened with a panel of monoclonal antibodies specific for amastigote-expressed proteins [18]. Two clones were identified, each recognized by a different monoclonal. Both of these monoclonals, VIIA4 and IIID4, recognized proteins expressed by the intracellular amastigote stages as detected by Western blots and flow cytometric analysis (Figure 2.1 A and B). Sequencing of these clones revealed that one encoded the previously identified flagellar $Ca2^{+}$ binding protein (FCaBP) that has been independently identified by several groups [25-27]. It has been reported that immunization with certain peptides from FCaBP, as well as the full-length protein, can induce partial protection against *T. cruzi* infection [28, 29], but its potential as a genetic vaccine candidate had not been addressed. For this reason, FCaBP was pursued in this study.

Sequencing of the second clone revealed that it shared extensive sequence homology with the β3 subunit of the AP-3 adaptor complex [30]. AP-3 β subunits have been characterized in a number of eukaryotic organisms but not in any of the protozoan parasites, therefore this gene was named *Tcb3* [Fralish and Tarleton, in preparation]. Based on the FACs analysis suggesting surface expression of the protein encoded by this

gene, the prediction that it is a single copy gene and the possibility that it is essential for parasite survival, we evaluated its potential as a genetic vaccine.

In this study we have also analyzed *LYT1* [14], a *T. cruzi* protein that was identified from a cDNA library using antibodies against the C9 component of the membrane attack complex (a complex formed as a result of complement activation which acts by creating a transmembrane pore leading to the lysis of the target cell)[31]. We hypothesized that this protein would be a good vaccine candidate primarily because it is secreted by the parasite early during host cell infection and would have access to the class I MHC processing pathway. *T. cruzi* peptides presented by the class I pathway are targets of $CD8^+$ T cells which are known to play a critical role in controlling *T. cruzi* infection [5, 32, 33].

Spleen cells from mice infected with *T. cruzi* recognize peptides derived from Tcb 3, FCaBP and Lyt1. CTL analysis was used to determine if peptides from Tcβ3, FCaBP, and LYT1 are presented to the host $CD8^+$ T cells during a natural infection, thus signifying immunogenicity. The three protein sequences were scanned for peptides containing motifs indicating potential binding to H-$2K^b$ the MHC haplotype of C57BL/6J, the mice used in these experiments. These peptides were synthesized and each used individually to stimulate spleen cells from chronically infected mice. After 6 days of in vitro stimulation the lytic activity of the resulting effector cells was evaluated against peptide-sensitized target cells. Effector cells lysed target cells pulsed with peptides from each of the three proteins as well as cells pulsed with PA14, a known *T.*

cruzi H-2K^b restricted CTL epitope from ASP-1 (ASP-$1_{509-516}$) [20], a but did not lyse the same cells pulsed with control peptide $OVA_{257-264}$ (Figure 2.2).

Heterologous expression. *FCaBP, Tcb3 and LYT1* were cloned into pCMVI.UBF3/2 (pCMV), a mammalian expression vector containing the cytomegalovirus (CMV) immediate-early gene promoter, a synthetic intron and a modified 3' untranslated region from human growth hormone (described in [12]). The resulting fusion proteins possess ubiquitin at the 5' end to enhance degradation by the proteosome and entry of the resulting peptides into the MHC class I presentation pathway. Expression of FCaBP and Tcβ3 was confirmed by antibody staining of transiently transfected COS-7 cells. Monoclonal antibodies specific for Tcβ3 or FCaBP positively stained COS-7 cells transfected with pCMV.*Tcb3* and pCMV.*FCaBP* respectively (Figure 2.3 C, D) but did not stain cells transfected with pCMV (Figure 2.3 A, B). Expression of LYT1 was not evaluated due to lack of a specific antibody.

Immunization elicits gene specific IFNg production. After confirmation that COS-7 cells transfected with the pCMV constructs expressed the proteins of interest, the plasmids were used to immunize mice. Mice received the empty CMV plasmid (pCMV), or pCMV.*Tcb3*, pCMV.*FCaBP* or pCMV.*LYT1*. In addition, animals in all groups received plasmids encoding GM-CSF and IL-12 as adjuvants (described in [12]). These cytokine adjuvants were co- administered in this experiment because they had been shown to enhance the *T. cruzi* specific immune responses generated by genetic immunization [12]. Mice were immunized and boosted six weeks later. Two weeks after

the boost, spleen cells were collected and analyzed by IFN-γ ELISPOT to determine if the immunizations generated memory or effector T cells that could recognize and respond, as indicated by IFN-γ production, to peptides from the corresponding proteins. IFN-γ production was assessed because it has been shown to be essential for controlling *T. cruzi* infection [34] and a vaccine that generates an IFN-γ response may be advantageous. Spleen cells from mice immunized with pCMV containing *Tcb3, FCaBP* or *LYT1* produced IFN-γ in response to overnight incubation with peptides from the respective proteins but spleen cells from mice immunized with pCMV alone did not respond to these peptides (Figure 2.4). These data indicate that immunizing with pCMV.*Tcb3*, pCMV.*FCaBP* or pCMV.*LYT1* elicited antigen-inducible cytokine responses.

Vaccination with pCMV.*LYT1* protects against *T. cruzi*-induced mortality. To determine if the immune responses generated by vaccination were capable of conferring protection, vaccinated animals were infected with a normally lethal dose of *T. cruzi*. As shown in Figure 2.5, immunizing with pCMV.*LYT1* and plasmids encoding IL-12 and GM-CSF enabled 80% of the infected mice to survive. However, mice immunized with pCMV.*FCaBP* in addition to cytokine adjuvants died by day 65 and immunization with pCMV.*Tcb3* plus cytokine adjuvants was no more protective than immunization with the control plasmid plus cytokine (an additional mouse from this group died on day 106 post infection).

In addition to monitoring protection against mortality, we were also interested in determining if vaccination was able to prevent or suppress disease development. In this

disease model, C57BL/6J mice infected with the Brazil strain of *T. cruzi*, disease develops predominantly in the skeletal muscle [35]. Therefore skeletal muscle was examined from each of the remaining groups on day 75 and 100 by histopathological analysis. At these time points all of the mice had similar and significant inflammatory responses in the skeletal muscle (data not shown). The remaining mouse from the pCMV.*Tcb3* immunized group died on day 106 post-infection and were survived by the two remaining pCMV.*LYT1* immunized animals. Tissues from the surviving pCMV.*LYT1* immunized mice were analyzed at one year post-infection but as demonstrated in Figure 2.5, inflammatory foci persisted. Thus, although immunization with pCMV.*LYT1* and cytokine encoding plasmids provided protection against mortality it did not prevent disease development under these experimental conditions.

Comparison of protection generated by single genes versus pools of genes from two *T. cruzi* gene families. As this study was underway reports were published demonstrating that immunizing with genes from the *T. cruzi* trans-sialidase (TS) gene superfamily protected mice against *T. cruzi*-induced mortality. The TS gene superfamily can be subdivided into two major subfamilies, the TS family composed of approximately 140 members 50% of which are enzymatically active, and the TS-like family consisting of several hundred members that are not enzymatically active [13]. Protection has been demonstrated by immunizing with an individual member of the TS subfamily [7], or individual TS-like genes, TSA-1 [8] and CRP [9]. In addition our laboratory showed that vaccinating with a combination of three TS-like genes, TSA-1, ASP-1 and ASP-2, greatly reduces the inflammation and necrosis normally associated with chronic Chagas disease

[12]. These data clearly demonstrate that members of the TS gene superfamily are good vaccine candidates. Therefore, we investigated the efficacy of immunizing with pools of either TS or mucin genes in comparison to a single TS-like gene, ASP-2, and the LYT1 gene. The mucin gene family, like the TS gene family, is a large gene family composed of several hundred members [13, 36, 37] that encode GPI-anchored surface proteins. Proteins expressed from these two gene families are the major components of the parasite surface coat and are well-characterized targets of the anti-*T. cruzi* immune response [13]. However the mucin genes have not been the focus of immunization studies. Based on these similarities and the protection generated from genetically immunizing with TS and TS-like genes it was hypothesized that mucin family members might also be good vaccine candidates.

The subset of TS and mucin genes studied were PCR amplified from amastigote cDNA with primers based on conserved sequences from a closely related subset of genes from each family. Multiple sequence alignments comparing TS and mucin genes sequences from GenBank revealed a group of approximately ten genes from each family that were similar enough to amplify using a simple set of PCR primers. The GenBank accession numbers of these genes are provided in Materials and Methods. The subset of TS superfamily genes studied was based on members of the TS subfamily, including TcTS2V0, TcTS 3.5, TcTS T4, TcTS 121, TcTS 154, TcTs 193, TcTS. The majority of mucin gene sequences in GenBank are from genes known to be expressed in either the epimastigote or the trypomastigote stage of the parasite lifecycle. The primers used in this study were based on selected trypomastigote expressed mucin genes, including EMUCt 2, 4 - 9, 11, 15, 17 and 18. The PCR amplified products were cloned into the

pCMV vector and the 5' ends of 20 clones were sequenced to confirm that a diverse pool of trans-sialidase and mucin genes had been amplified. Mice were vaccinated with pCMV alone, pCMV.*ASP2* (a TS-like gene that had been previously shown to induce protection [12]; note that this sequence would not be expected to be included in the TS mix), pCMV.*LYT1*, pCMV.*TS mix* or pCMV.*Mucin mix* (pCMV containing a mixture of trans-sialidase or mucin genes respectively). In order directly assess the level of protection generated by immunization with these genes, cytokine adjuvants were not co-administered.

As shown in Figure 2.7, 100% of the mice immunized with the vector alone died by 60 days post-infection. In contrast, 80% of the mice immunized with pCMV.*ASP2* were protected, confirming previously reported results [12]. Immunization with pCMV.*LYT1* and pCMV.*TS mix* protected 75% of the animals whereas mice immunized with pCMV.*Mucin mix* were not protected; only 25% survived until 75 days post-infection.

Discussion

Justification for vaccine development against *T. cruzi* infection has been bolstered by the mounting evidence linking parasite persistence to disease development (reviewed in [3]) as well as the increasing understanding of immune mechanisms important in the control of the infection. Antibodies, $CD8^+$ T cells and a type-1 $CD4^+$ T cell response characterized by IFN-γ production are essential for controlling *T. cruzi* infection in mice [5, 32, 33, 38, 39]. Genetic vaccination has been shown to generate both humoral and cell-mediated immune responses (reviewed in [4]) and has been shown to be an effective

means of generating protective responses against *T. cruzi* infection in the murine model [7-12, 40]. A majority of the *T. cruzi* genes tested as genetic vaccines are members of the TS gene superfamily, TS, TSA-1, ASP-1, ASP-2 and CRP [7-10, 12]. Although these data validate genetic vaccination as a means to control *T. cruzi* induced mortality, a genetic vaccine candidate(s) has still not been identified that can protect 100% of animals from lethal infection nor protect against subsequent development of chronic disease. In addition, an effective field vaccine will have to protect a diverse human population from infection with a diverse parasite population and thus will probably require a complex mixture of genes. Therefore there continues to be a need for identification and testing of additional vaccine candidates.

The goals of this study were to 1) identify and evaluate genes as candidate vaccines that were not members of the large multi-gene families and 2) evaluate expanded numbers of family member genes by using pools of genes from superfamilies. The rationale for evaluating genes outside of the large gene superfamilies was based on the likelihood that not all members of the large gene families would be expressed in all strains of the parasite, and that the fervent immune response generated against large-gene family members may mask other important immunological targets. Additionally the possibility that the conservation of related epitopes in gene family members may subvert the immune response by acting as altered peptide ligand antagonists has been a concern [41, 42]. However, as many TS superfamily genes have been shown to be effective genetic vaccines we chose to evaluate the efficacy of immunizing with an expanded pool of a subset TS genes including enzymatically active family members. A selected sub-group of TS genes might cover those expressed by many parasite strains, provide

multiple targets but perhaps not the overwhelming number presented by *T. cruzi.* In addition, mucin genes were included because they are the other large gene family that is a well-characterized target of immune responses, are also surface expressed but have not been the focus of immunization studies.

Tcb3, a novel *T. cruzi* gene, and *FCaBP,* were identified by screening an amastigote cDNA expression library with anti-*T. cruzi* monoclonal antibodies. Although the FCaBP had been previously identified [25-27] and shown to elicit protective responses in mice immunized with the protein [29], its efficacy as a genetic vaccine has not been previously reported. This protection is significant because protein immunization would not be expected to efficiently prime cytotoxic T lymphocytes, which are an important component of protection. Antibodies against both Tcβ3 and FCaBP bind to intact amastigotes indicating that these proteins are exposed on the parasite surface. We were interested in pursuing surface proteins because they are normally excellent targets of Ab responses and because of their potential to also be released from the parasite surface where they may gain access to the MHC class I processing pathway and stimulate $CD8^+$ T cell responses. *LYT1* [14] was included in this study based on the hypothesis that it too may gain access to the host cell class I MHC processing pathway since it is secreted early in the process of *T. cruzi* invasion of host cells.

Peptides from each of these three proteins sensitized target cells for lysis by spleen cells isolated from chronically infected mice, thus confirming that these proteins are naturally processed and presented to host $CD8^+$ T cells during the couse of *T. cruzi* infection. However despite this fact as well as the demonstration that immunization with the *FCaBP, LYT1* and *Tcb3* elicited potent cell-mediated immune responses, only

pCMV.*LYT1* generated substantial protection. These data demonstrate that not all targets of the $CD8^+$ T cell response in *T. cruzi* are equally effective as genetic vaccines.

Two pools of genes were tested as genetic vaccines. These pools contained a subset of TS or mucin gene family members. Together mucin and TS molecules make up the majority of the surface coat of the *T. cruzi* [13]. Both the TS and mucin gene superfamilies consist of several hundred members. A small percentage of TS superfamily members are catalytically functional trans-sialidases which transfer host derived sialic acid to the surface expressed sialic acid acceptors. Since *T. cruzi* can not synthesize sialic acid *de novo*, this is the sole means by which sialization of surface molecules, like the mucins, occurs. Sialic acid is thought to play a role in infectivity and survival in the mammalian host [43]. Immunization with members of the TS gene superfamily has been shown to elicit protection in mice against a lethal dose of *T. cruzi* [7-10, 12]. Because most of these genes members of the enzymatically inactive TS-like subfamily, we were interested in evaluating the efficacy of vaccinating with pools of genes from the TS subfamily, which contains fewer members, half of which are enzymatically active. In addition, because of the protection attained with TS superfamily members and the similarity between expression and localization mucin and TS proteins, we also evaluated vaccinating with pools of mucin genes.

Immunization with the pool of TS genes generated a significant protective response in mice against normally lethal *T. cruzi* infection. The level of protection generated by immunizing with the pool of TS genes was similar to that attained with immunizing with a single TS-like molecule, ASP-2, a gene that had been previously reported to induce protection [12]. This is in contrast to the high mortality found when

mice were immunized with a pool of genes from the *T. cruzi* mucin family, only 25% survived 75 days of infection. These data indicate that although the mucin and TS gene superfamilies are both extremely large gene families that encode abundantly expressed GPI-anchored surface proteins, the members tested in this study differ in their efficacy as genetic vaccines. This may be related to the high level of glycosylation that is found on mucin molecules that may mask immunogenic epitopes or inhibit processing and presentation of these molecules. Although the level of protection was similar between mice immunized with a single TS-like member, ASP-2, and with a pool of TS members, the advantage of immunizing with pools of genes may become more evident if tested in a diverse set of mouse strains against a diverse set of parasite strains.

The data presented herein demonstrate genetic immunization with either *LYT1* or a pool of TS genes generates a protective immune response against normally lethal doses of *T. cruzi*, whereas a pool of mucin genes was not capable of inducing protection. These results validate the efficacy of using gene pools as vaccines for *T. cruzi.* Based on these results it would be interesting to determine if immunizing with even more complex combination genes, for example a mixture of LYT1, TS-like and TS genes, elicits a higher level of protection.

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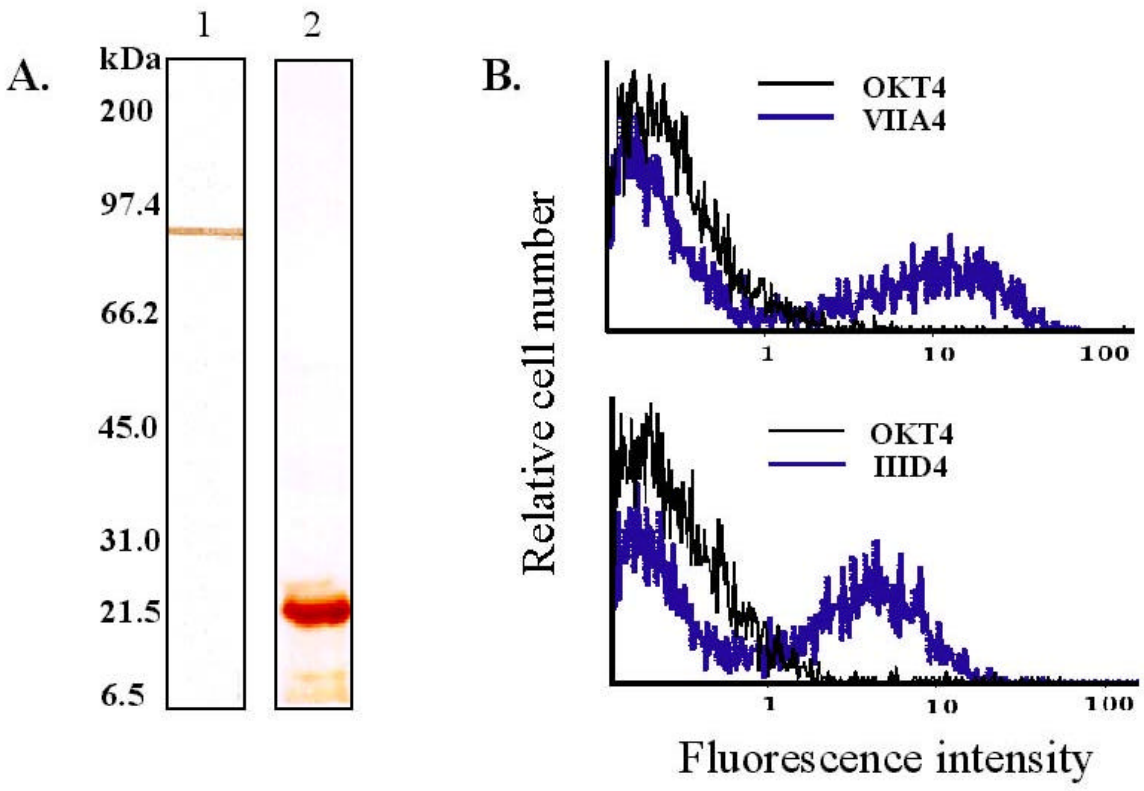

Figure 2.1. Detection of amastigote proteins by Western blot (A) and flow cytometric analysis (B). A. Amastigote lysates were separated by SDS-PAGE, proteins were transferred to nitrocellulose membranes and blotted with mAb VIIA4 (1) and IIID4 (2). B. Live amastigotes were stained with mAb VIIA4, IIID4 and OKT4, a negative control antibody used to indicate background staining, followed by staining with a secondary FITC-labeled antibody. These cells were then analyzed by flow cytometry on a Coulter Elite Analyzer (Beckman Coulter, Inc.).

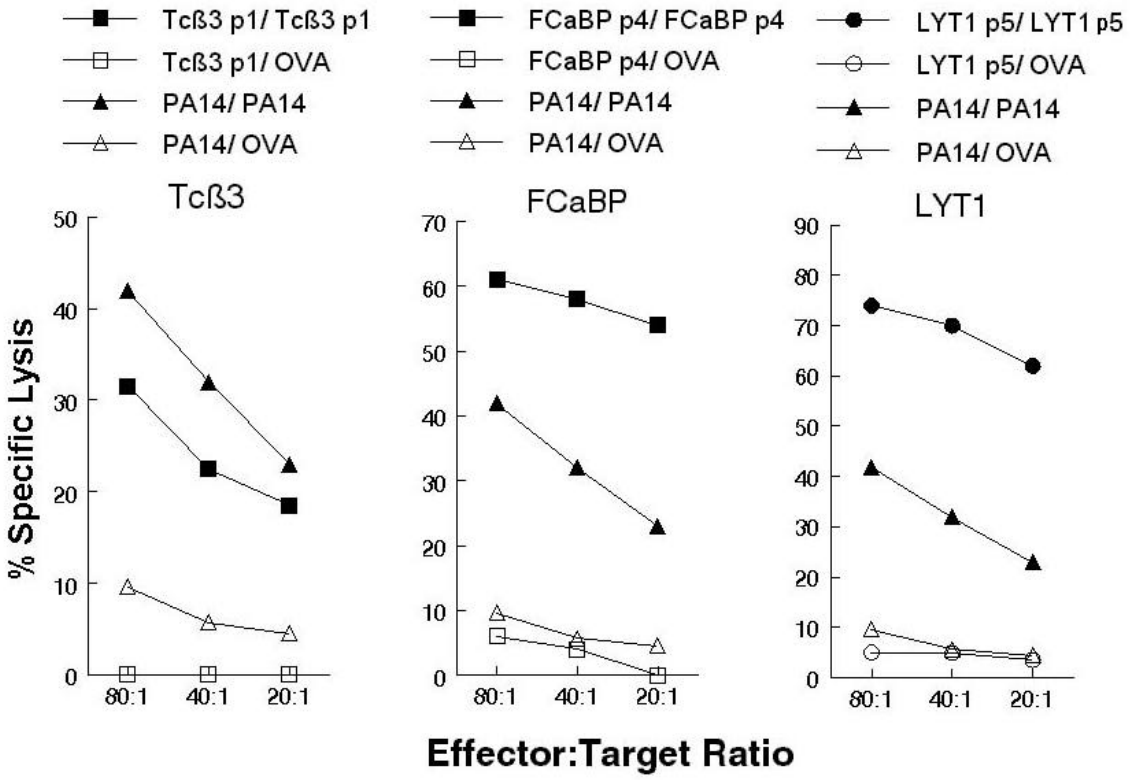

Figure 2.2. CTL analysis of spleen cells from chronically infected mice incubated with ^{51}Cr-labeled target cells pulsed with synthetic peptides from the three gene products. The three protein sequences were scanned for peptides containing motifs indicating potential binding to H-2K^b one of the class I MHC alleles of C57BL/6J, the mice used in these experiments. These peptides were synthesized and used to stimulate spleen cells from chronically infected mice in vitro for 6 days. The resulting effector cells were incubated with ^{51}Cr-labeled H-2^b target cells (RMA-S) pulsed with the matching peptide or a negative control peptide OVA, a H-2K^b restricted chicken ovalbumin CTL epitope OVA$_{257-264}$, for five hours. PA14 is a known H-2K^b restricted CTL epitope from ASP1, a trans-sialidase gene family member from *T.cruzi* [20].

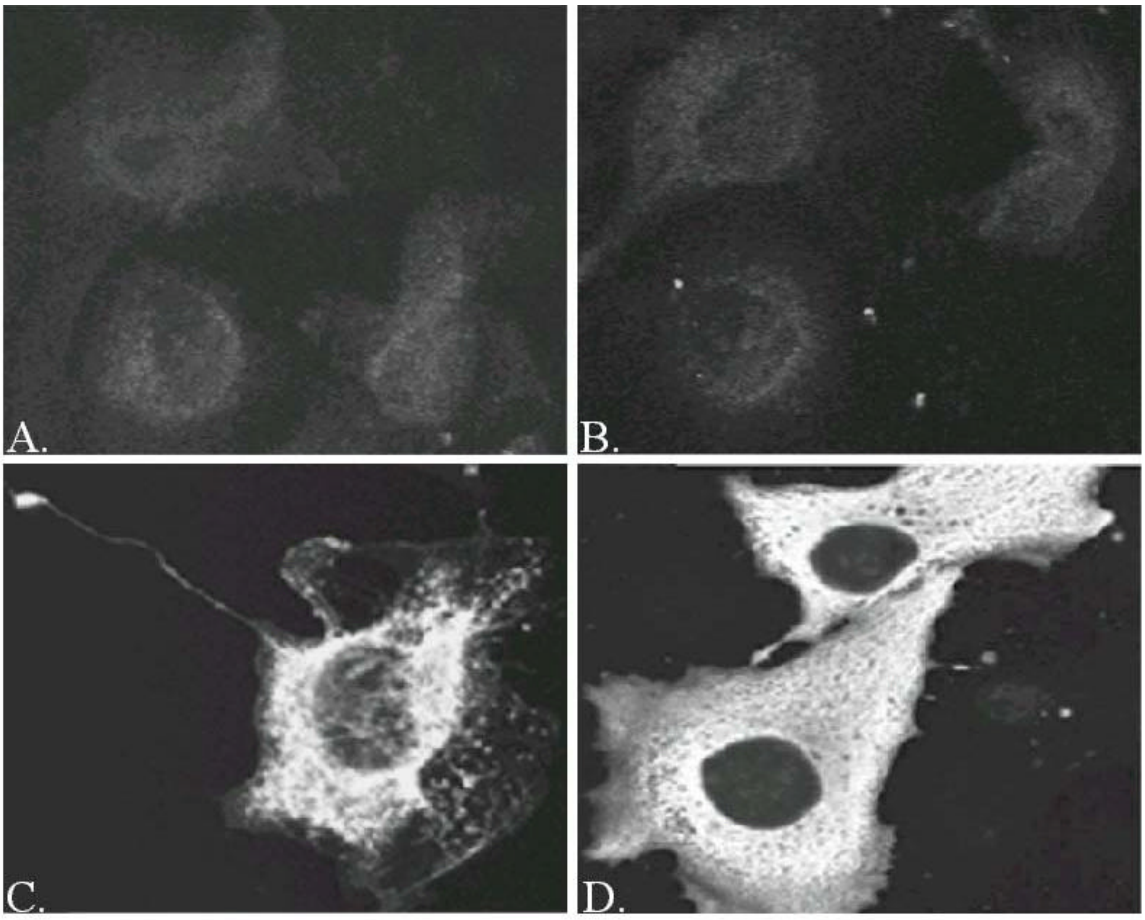

Figure 2.3. Immunofluorescent staining of COS-7 cells transfected with pCMV plasmid (A, B), pCMV.*Tcb3* (C), or pCMV.*FCaBP* (D). *Tcb3* and *FCaBP* were cloned into the pCMVI.UBF3/2 mammalian expression vector and expression from these plasmids was assessed by immunofluorescence. Cells in panels A and C were stained with the anti-Tcβ3 mAb VIIA4 and panels B-D with the anti-FCaBP mAb IID4 all cells were then stained with FITC-labeled goat anti-mouse Ig-G.

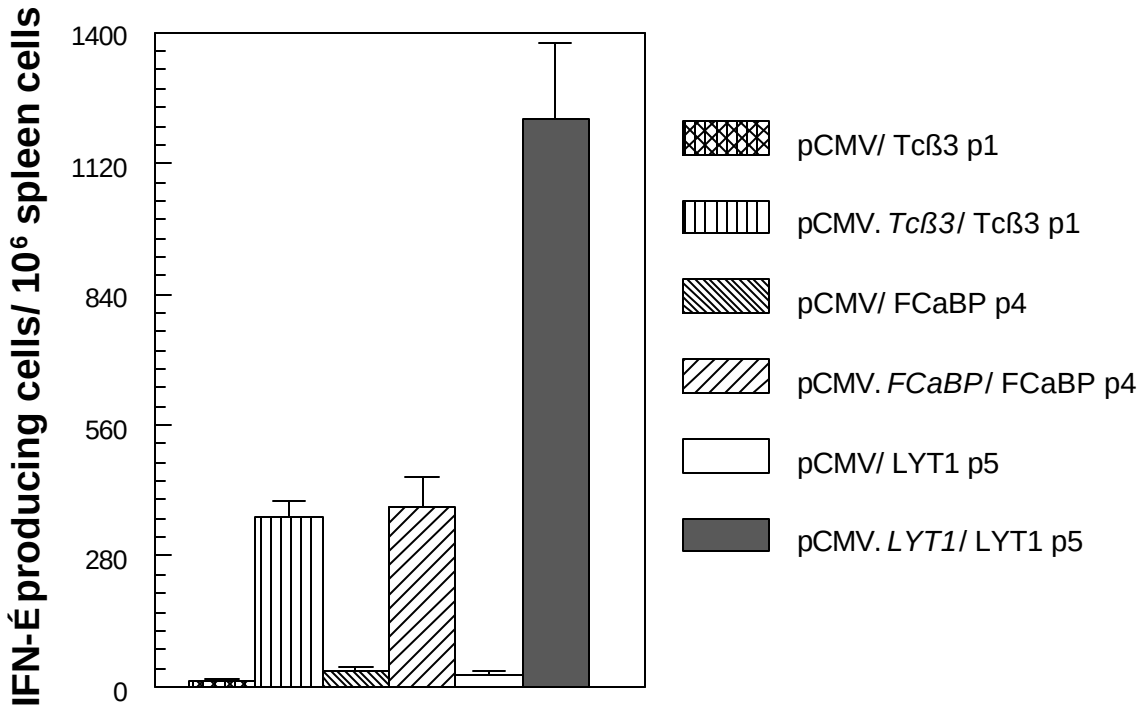

Figure 2.4. ELISPOT analysis of IFN-g producing cells induced by genetic immunization. Spleen cells from immunized mice were collected two weeks following the second immunization and plated in triplicate +/- peptides (Tcβ3 p1, FCaBP p4 and LYT1 p5 respectively) overnight in 96-well nitrocellulose bottomed plates previously coated with rat anti-mouse IFN-γ. The plates were washed and a rabbit anti-mouse IFN-γ pAb was added for two hours followed by a goat α-rabbit peroxidase labeled antibody. Spots were visualized after addition of substrate and counted on a dissecting scope. The mean of the number of spots generated when spleen cells were incubated with media alone were subtracted from the mean of the number of spots obtained when those spleen cells were incubated with respective peptides. Legend indicates immunization plasmid followed by peptide.

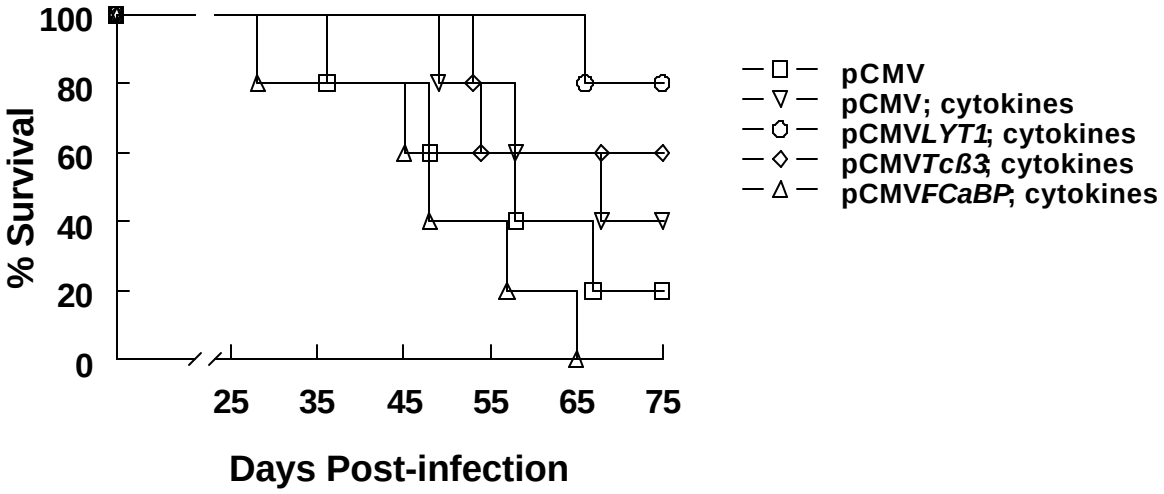

Figure 2.5. Immunization with pCMV.*LYT1* protects mice from normally lethal *T. cruzi* infection. C57BL/6J, 5 mice per group, were immunized with 25 µg of pCMV, pCMV.*Tcb3*, pCMV.*FCaBP* or pCMV.*LYT1* in addition to 25 µg of cytokine plasmids encoding IL-12 and GM-CSF (pcDNA3.msp35, pcDNA3.msp40 and pCMVI.GM-CSF). Immunizations were repeated at six week and mice were infected intraperitoneally two weeks later with 10^5 blood form trypomastigotes. Where possible, one mouse per group was sacrificed for histopathology on days 75 and 100. An additional mouse immunized with pCMV.*Tcb3* died on day 106. Mortality was assessed daily.

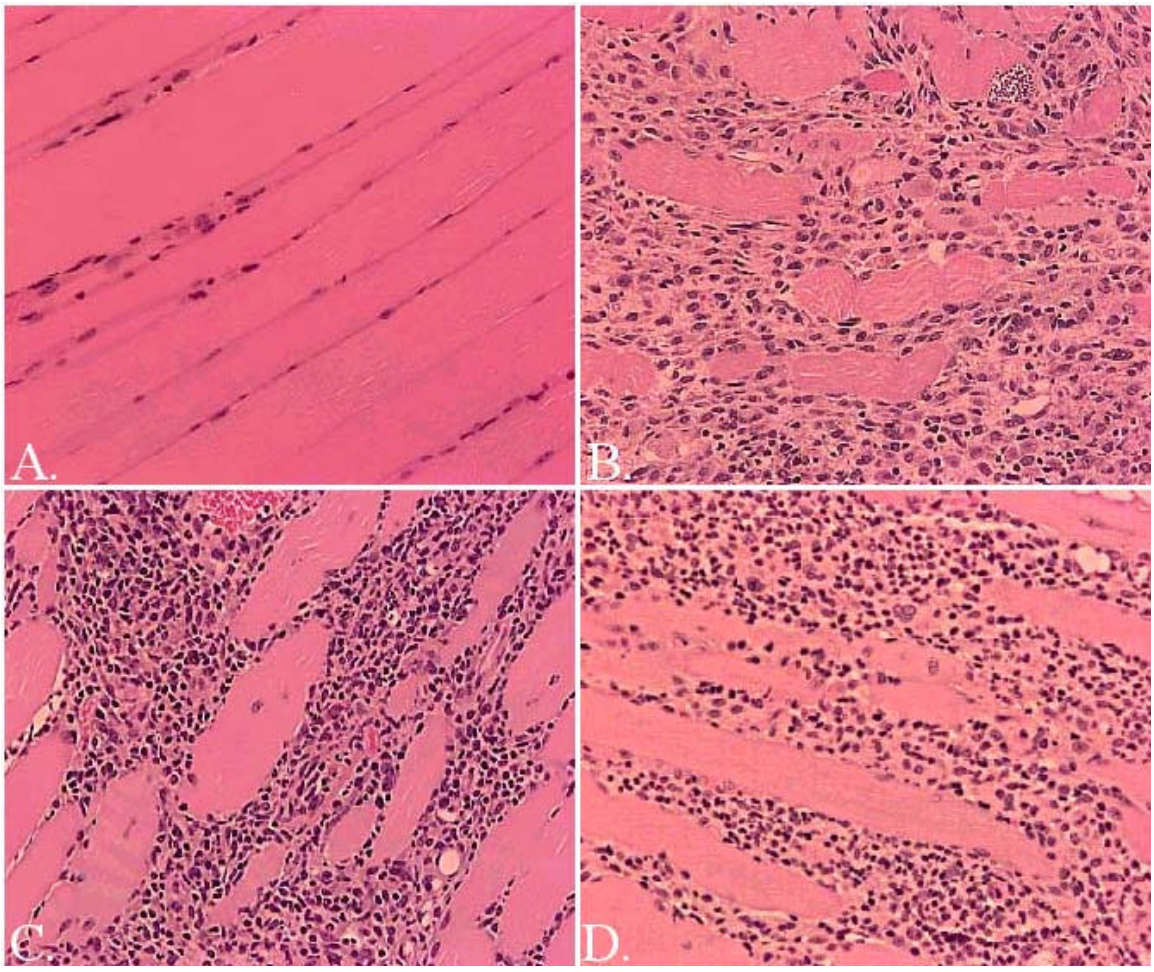

Figure 2.6. Inflammation persists in mice immunized with pCMV.*LYT1* and cytokine adjuvants. Hematoxylin and eosin stained skeletal muscle sections from a uninfected C57BL/6J mouse (A) and mice immunized with pCMV.*LYT1* at 75 d.p.i (B), 100 d.p.i. (C) and 365 d.p.i. (D).

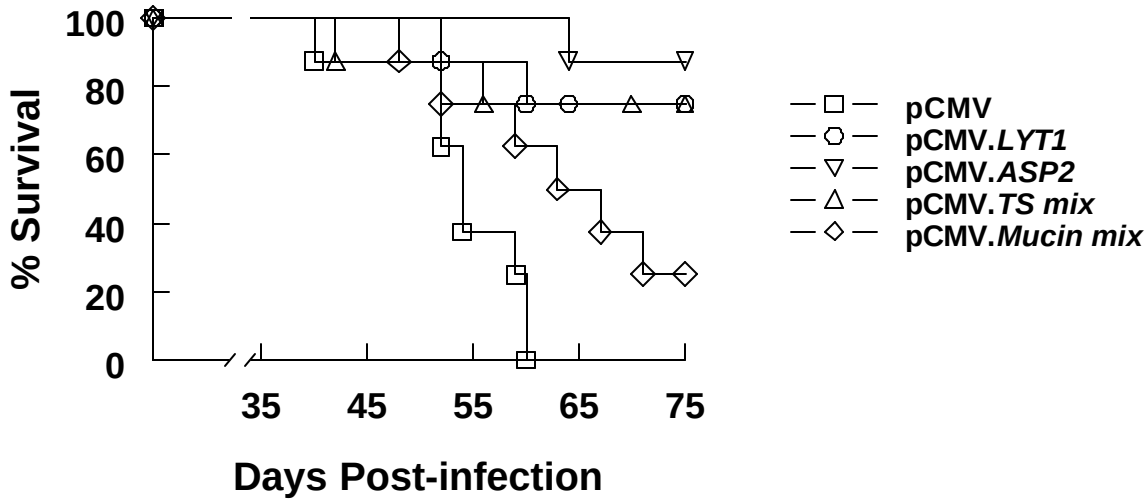

Figure 2.7. Immunizations with pCMV.*LYT1* or a mixture of trans-sialidase genes without adjuvant protects mice from normally lethal infection with *T. cruzi.* C57BL/6J, 8 mice per group, were immunized with 25 µg of pCMV, pCMV.*LYT1*, pCMV.*ASP2* (a trans-sialidase gene previously shown to induce a protective response, used as a positive control) or 100 µg of pCMV.*TS mix* and pCMV.*Mucin mix*. Immunizations were repeated at six weeks and two weeks later the mice were infected with 2.5 x 10^5 blood form trypomastigotes. Mortality was assessed daily.

CHAPTER 3

β3 ADAPTIN EXPRESSION IS ESSENTIAL FOR NORMAL GROWTH AND CELL DIVISION IN TRYPANOSOMES[2]

[2] Fralish, B. H. and R. L. Tarleton. To be submitted to *Molecular and Biochemical Parasitology*.

Abstract

Adaptor protein complexes are integral components of vesicle-mediated protein trafficking in the endocytic and secretory pathways, recruiting both the coat protein as well as vesicle cargo. To date four adaptor protein complexes have been identified, AP-1-AP-4. Although the subunits of these heterotetrameric complexes share significant homology, their trafficking routes are slightly different. AP-2 coated vesicles initiate at the plasma membrane while vesicles coated with AP-1, AP-3 or AP-4 bud from the trans-Golgi network and/or endosomes. These complexes have been elucidated in a number of eukaryotic organisms but have not yet been functionally characterized in any of the protozoans. By screening a cDNA expression library from *Trypanosoma cruzi* we have identified a novel *T. cruzi* gene, Tcβ3, that is 55% similar and 35% identical to the N-terminal domain of the human β3A adaptin, one of the large subunits of the AP-3 complex. Endogenous Tcβ3 is localized to a vesicular/punctate compartment throughout *T. cruzi* epimastigotes; a pattern that can be directed by the first 834 bp of *Tcb3* as evidenced by expression of GFP and myc tagged constructs. These results further support the conclusion that Tcβ3 is a functional adaptin molecule. However GFP and myc expression was not detected in parasites transfected with constructs containing either the first 1 kb or the entire 2.7 kb *Tcb3* open reading frame. In addition we were unable to obtain stable transfectants expressing the 0.834 kb tagged constructs. These results suggest Tcβ3 fusion proteins are acting as dominant negative mutants and in combination with our failure to generate single or double knockouts parasites indicate that Tcβ3 is essential for parasite survival. Further functional characterization was achieved by using the Tcβ3 sequence to identify the N-terminal region of a β3 homologue from *T. brucei*

and analyzing the function of the β3 like protein in *T. brucei* by RNAi**.** Expression of dsRNA specific for the *T. brucei*-β3 homologue led to growth inhibition, flagellar detachment and a block in cytokinesis in *T. brucei* procyclic cells. These results confirm that β3-like proteins play an essential role in trypanosomes.

Introduction

In our search for potential vaccine candidates we identified a novel *T. cruzi* gene that shares extensive homology to the human β3A (NP_003655) subunit of the AP-3 adaptor complex. Although this gene did not prove to be an effective genetic vaccine (Chapter 2) we were intrigued by its potential role in protein trafficking within the parasite.

Adaptor protein complexes are integral components of vesicle-mediated protein trafficking in the endocytic and secretory pathways, recruiting and specifying both the coat protein and vesicle cargo (reviewed in [1]). Although four heterotetrameric adaptor protein complexes have been characterized in higher eukaryotes, AP-1- AP-4 [2-5], each consisting of two large subunits (γ/α/δ/ε and β1-β4 ~90-130 kDa) one medium subunit (μ1-μ4 ~50 kDa) and one smaller subunit (σ1-σ4 ~20 kDa) [5, 6], very little is known about these complexes in the kinetoplastids.

The kinetoplastids, a group of flagellated protozoa named for their single kinetoplast which houses the mitochondrial DNA, include the causative agents of Chagas disease, African sleeping sickness and leishmaniasis, *T. cruzi*, *T. brucei* and *Leishmania* respectively. Although receptor-mediated endocytosis and coated vesicles have been documented in these organisms the adaptor and coat proteins involved in these processes have not been functionally characterized [7-14]. In fact only recently was the first adaptin homologue, a β1-like protein from *T. brucei*, identified from these organisms and its function has not yet been determined [15].

Endo- and exocytosis in the kinetoplastids occurs exclusively via the membrane of the flagellar pocket. Fusion of cytoplasmic vesicles with the plasma membrane is

blocked by the presence of a cortex of more than 100 closely spaced microtubules that lie directly beneath the plasma membrane. However these microtubules are absent beneath the membrane of the flagellar pocket, apparently allowing for vesicular trafficking at the flagellar pocket membrane domain [16, 17].

The possibility that the structural constraints imposed by the unique cytoskeletal architecture of these organisms may necessitate novel mechanisms of protein trafficking validates further investigation of proteins involved in these processes. In this study we identified and characterized a novel *T. cruzi* protein, Tcβ3, which is homologous to the human and Drosophila β3 adaptins of the AP-3 adaptor protein complex [1]. We used the sequence homology between Tcβ3 and other β3 adaptins to clone the conserved N-terminal domain from *T. brucei* and have shown that generation of dsRNA specific to this gene leads to a significant growth defect, loss of flagellar adhesion and a block in cytokinesis.

Materials and Methods

Trypanosomes. Epimastigotes of *T. cruzi* (CL strain) were cultured at 26°C in liver infusion tryptose (LIT) media supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone, Logan, UT). Trypomastigotes and amastigotes of *T. cruzi* were maintained in vitro via passage of tissue culture-derived trypomastigotes in monolayers of Vero cells (African green monkey kidney cells; American Type Culture Collection, ATCC, Rockville, MD) maintained in RPMI-1640 medium (Mediatech, Herndon, VA) containing 10% heat-inactivated FBS (HyClone), 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 50 μg/ml gentamycin (all from

Gibco BRL, Gaithersburg, MD) [18]. Procyclic forms of the *T. brucei* 29-13 strain (kindly provided by Drs. Elizabeth Wirtz and George Cross) which contain integrated copies of T7 RNA polymerase and the tetracycline repressor (respectively maintained by culturing in the presence of 15 µg/ml G418 and 50 µg/ml hygromycin) were grown in SM media supplemented with 15% heat inactivated FBS (HyClone), 7.5 µg/ml bovine hemin (Sigma, St. Louis, MO) and penicillin-streptomycin 100 units/ml and 100 µg/ml respectively (Gibco BRL).

cDNA library screening. *T. cruzi* specific monoclonal antibodies, generated as described [19], were used to screen a *T. cruzi* amastigote cDNA expression library (cloned into Uni-ZAP XR expression vector, Stratagene, La Jolla, CA) also described in [19]. Fifty plates (150 mm) containing a total of 10^5 plaque-forming units were screened with various monoclonal antibodies. Three rounds of screening were performed for each positive plaque before in vivo excision of the pBluescript II (SK-) containing insert from Uni-Zap XR according to the manufacturer's instructions (Stratagene, La Jolla, CA).

Western blots. *T. cruzi* amastigotes were harvested from Vero cell cultures, resuspended at 10^9/ml in lysis buffer (10 mM Tris-HCL, pH 7.5; 5mM EDTA; 1% Nonidet P-40; 1 mM phenyl-methanesulfonyl fluouride; 10 mg/ml aprotinin; 50 U/l trasylol; 10 mg/l leupeptin) and exposed to repeated freeze-thaw treatment. The lysates were cleared by centrifugation (30 minutes, 4°C at 14,000 x *g*), supernatants collected and separated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) by semi-dry

electroblotting for 2 hr at 20 V at room temperature in Bjerrum and Schafer-Niesen buffer (48 mM Tris base; 39 mM Glycine; 200 ml/l methanol; 0.04% SDS)[20]. Membranes were blocked in phosphate buffer saline (PBS; 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 8 mM KCl, pH 7.4) containing 1% non-fat dry milk, 0.1% Tween-20, incubated with respective monoclonal antibodies for 1 hr at room temperature, and then with a horseradish peroxidase (HRP) labeled goat anti-mouse IgG and IgM (Accurate Chemical and Scientific Corp., Westbury, NY), 1 hr at room temperature. Blots were developed with 1.6 mM 3,3' diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M Tris-HCL pH 7.6 containing 0.03% H_2O_2 at room temperature until stopped with H_2O.

Flow cytometric analysis. Amastigotes harvested from infected Vero cells were washed in PAB (0.1% BSA, 0.1% sodium azide in PBS), resuspended at 10^6/ 50 µl in PAB containing the mAb culture supernatants (diluted 1:1) and incubated for 30 minutes at 4°C. OKT4, a monoclonal antibody that recognizes human helper T-cells (ATCC-CRL 8002), was used as a negative control. The cells were then washed in PAB, stained for 30 minutes at 4°C with fluorescein isothiocyanate (FITC)-labeled goat $F(ab')_2$ anti-mouse IgG (Pharmingen, San Diego, CA; diluted 1:50 in PAB) washed again in PAB and then analyzed by flow cytometry on an Coulter Elite Analyzer (Beckman Coulter, Inc., Fullerton, CA).

DNA sequencing. The cDNA clones were sequenced by the Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia, using the Taq dye terminator chemistry on an ABI 373 DNA sequencer (Foster City, GA). Sequence homology searches of the database maintained at the National Center for Biotechnology Information (NCBI; Bethesda, MD) were carried out using the BLASTP and BLASTN algorithms at the NCBI web page (http://www.ncbi.nlm.nih.gov/BLAST/).

Immunofluorescence. Parasites were washed 2 times in PBS, allowed to settle onto poly-L-lysine (Sigma) coated slides and fixed in ice-cold methanol for 20 min. The cells were blocked with 5% BSA in PBS for 1 hr. and stained with hybridoma supernatants or an anti-myc antibody (Invitrogen, Carlsbad, CA) for 1 hr. OKT4 (mouse anti-human CD4) and chronic mouse serum were used as negative and positive controls, respectively. Following 3 washes in PBS, the cells were incubated with FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (Pharmingen) for 1 hr. After 3 washes in PBS the slides were mounted in 10% glycerol, 2.5% DABCO (1,4-diazabicyclo- (2.2.2)-octane, Sigma) containing 0.5 µg/ml 4', 6-diamidino-2-pehnylindole (DAPI, Molecular Probes, Eugene, OR). Parasites expressing green fluorescent protein constructs were fixed in 2.5% paraformaldehyde for 20 min at 4 °C, washed 2 times in PBS and permeabilized for 2 minutes in 0.01% saponin containing 0.5 µg/ml DAPI washed again and mounted as described above. Cells were imaged on a DMIRBE microscope (Leica Micro systems, Wetzlar, Germany) using OpenLAb software (Improvision, Coventry, UK).

Plasmid construction. *Tcb3-GFP constructs.* Tcβ3-GFP fusion constructs were created by ligating the first 0.834 kb, 1.122 kb or the entire *Tcb3* open reading frame (excluding the stop codon) to GFP and inserting these ligation products into pRIBOTEX [21], the resulting plasmids were named pRT-0.8*Tcb3-GFP*, pRT-1.1*Tcb3-GFP*, pRT-2.7*Tcb3-GFP*. Specifically, the first 0.834 kb, 1.122 kb and full-length *Tcb3* were PCR amplified using the a forward primer introducing a BamH I site (underlined), 5'-CGCGGATCCATGAGTCGTGCGATAATTG-3' and individual reverse primers each encoding a EcoR I site (underlined), 5'-CGGAATTCTGTATGCTTCGTTAAACA-3' (Tcβ3 nucleotides 817-834), 5'-CGGAATTCGGCGCAATGATAAAACAA-3' (Tcβ3 nucleotides 1105-1122) and 5'-CGGAATTCTGACATTCTCTCCTCGTGCTC-3'(Tcβ3 nucleotides 2671-2691). A modified GFP gene without the start codon was PCR amplified from pBIN 35S-*mgfp5*-ER plasmid DNA [22, 23] using forward and reverse primers encoding EcoR I and Xho I sites respectively (underlined), 5'-CCGGAATTCAGTAAAGGAGAAGAACTT-3' and 5'-CCGCTCGAGTTATTTGTATAGTTCATC-3'. Tcβ3 and GFP PCR amplified products were digested with the respective restriction enzymes and ligated using T4 DNA ligase (New England Biolabs, NEB, Beverly, MA) for 30 min at room temperature. After 30 minutes, BamH I and Xho I digested pRIBOTEX was added and the reaction was incubated for an additional 2 hr at RT. A control plasmid, pRIBOTEX.*GFP*, was generated by PCR amplifying the GFP gene from pBIN 35S-*mgfp5*-ER plasmid DNA using a forward primer that included its start codon and incorporated an EcoR I site (underlined), 5'-CCGGAATTCATGAGTAAAGGAGAAGAACTT-3', and the same

GFP reverse primer previously described. This PCR product was digested with EcoR I and Xho I and ligated into pRIBOTEX that had been similarly digested.

Tcb3-myc tagged constructs. *Tcb3*-myc tagged constructs were generated by PCR amplifying the first 0.834 kb, 1.122 kb or the entire *Tcb3* open reading frame (excluding the stop codon) using a forward primer that incorporates a BamH I site (underlined), 5'-CGCGGATCCATGAGTCGTGCGATAATTG-3' and reverse primers that incorporate the myc epitope tag (bold) followed by a stop codon and an EcoR I site (underlined), 5'-CCGGAATTCTTA**CAGATCCTCTTCTGAGATGAGTTTTTGTTC**TGTATGCTTCGTTAAACA-3' (Tcβ3 nucleotides 817-834), 5'-CCGGAATTCTTA**CAGATCCTCTTCTGAGATGAGTTTTTGTTC**GGCGCAATGATAAAACAA-3' (Tcβ3 nucleotides 1105-1122) 5'-CCGGAATTCTTA**CAGATCCTCTTCTGAGATGAGTTTTTGTTC**TGACATTCTCTCCTCGTGCTC-3'(Tcβ3 nucleotides 2671-2691). These PCR amplified products were digested with BamH I and EcoR I and inserted into pRIBOTEX by directional cloning. Resultant plasmids were named pRT-0.8*Tcb3myc*, pRT-1.1*Tcb3myc*, pRT-2.7*Tcb3myc*.

pZJM.EGFP-puro (pZJM.Ep) and pZJM.Ep-no2T7. Replacement of the phleomycin drug resistance gene in pZJM [24] with the EGFP-puromycin chimeric marker gene from pEGFP-puro [25] was achieved in several steps. The phleomycin drug resistance gene was released from pZJM by digesting with Nco I, this digestion also releases the actin 3' UTR and part of the rDNA spacer. These features were reconstituted by PCR amplification from pZJM plasmid DNA with a primer specific for the rDNA spacer region that included the Nco I site (underlined), 5'-

AACTTCCATGGTACAAACTGGTCG-3', and a primer specific for the actin 3' UTR that also included the last 28 bp of the puromycin resistance gene (bold) containing BamH I site (underlined), 5'-

CCGGGATCCACCGGATCTAGATAACTGAGCCAAAATTGTTCTGTAG-3'.

This PCR amplified product was digested with Nco I and Bam HI. The EGFP-puromycin chimeric marker gene from pEGFP-puro was isolated by digestion with Nco I and BamH I releasing all of the chimeric gene except the most C-terminal 20 bp which were reconstituted by the primer used to PCR amplify the actin 3' UTR from pZJM. The Nco I/BamH I digested EGFP-puro fragment and the Nco I/BamH I digested actin 3' UTR-rDNA spacer fragment were ligated into Nco I digested pZJM. The resulting plasmid was named pZJM.EGFP-puro, (pZJM.Ep). pZJM.Ep-*no2T7*, a control plasmid lacking the opposing T7 promoter cassette, was generated by digesting pZJM.Ep with Kpn I and BamH I, blunting the ends with T4 DNA polymerase (NEB) and ligating with T4 DNA ligase (NEB).

pZJM.Ep-Tbb3. pTOPO-*Tbb3* (generated as described below) was digested with Hind III and Xho I, using the vectors' Hind III site and the inserts 3' Xho I site, the insert was ligated into Hind III/Xho I digested pZJM.Ep. The resulting construct was named pZJM.Ep-*Tbb3*.

Recombinant plasmids were transformed into *E. coli* DH5-alpha competent cells and grown in Luria broth (LB) supplemented with 100 µg/ml ampicillin. Plasmid constructs were confirmed by restriction analysis and DNA sequencing

Identification of *T. brucei* b 3 adaptin. Primers were designed based on multiple sequence alignments of the *Trypanosoma cruzi* beta-3 adaptin like gene (*Tcb3*), *Homo sapiens* beta-3A-adaptin (NM_003664), *Homo sapiens* beta-3B (NM_004644) and the *Drosophila melanogaster* β3 (AE003431) gene sequences. These primers, 5'-CCGATGTAGTGAAGAAC-3' and 5'-CCGCTCGAGACCACCTGCCCCCACTC3' (Xho I site underlined) amplified a 492 nt product from procyclic *T. brucei* cDNA. Briefly, RNA was isolated from 5 x 10^8 procyclics using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to manufacturers instructions. 5 μg of RNA was used for cDNA synthesis with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) following manufacturers guidelines. The PCR amplified product was cloned into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced. The resulting construct was named pTOPO-*Tbb3*.

Transfection. Procyclic *T. brucei* 29-13 cells were grown to a density of 1 x 10^7/ml and harvested by centrifugation for 5 min at 1000 x g. Cells were washed with and resuspended in cytomix buffer [26] at 10^8/ml. 20 μg of linearized DNA (Not I digested) and 400 μl of cells were added to 4 mm gap electroporation cuvettes (BTX, Gentronics, Inc., San Diego, CA) and electroporated with one pulse in a BTX electroporator with peak discharge at 1.5 kV in resistance timing mode R2 (24 ohms). Immediately following electroporation cells were transferred to 9.5 mls of SM medium containing 15 μg/ml G418 and 50 μg/ml hygromycin to maintain the T7 RNA polymerase and the tetracycline repressor genes respectively. Puromycin, 10 μg/ml, was added 16 hr later. Cells were cultured in the presence or absence of 1 μg/ml tetracycline added either 16 hr

post-transfection or 3 weeks after puromycin drug selection as indicated in figure legends. Growth curves were calculated by counting cells every 24 hours after initiating cultures at 10^6 cells/ml, when cells exceeded 5 x 10^6 cells/ml they were split back to 10^6 cells/ ml and counts were adjusted by respective dilution factors. *T. cruzi* epimastigotes were grown in LIT media to a density of 2 x 10^7/ml harvested by centrifugation (1000 x g), washed with and resuspended in ice-cold electroporation buffer (PBS with 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$) [27] at 4 x 10^8/ml. 50 µg of DNA and 400 µl of cells were added to 0.2 cm Gene Pulser cuvettes (BIO RAD, Hercules, CA) with two pulses at 400V and 500 microfarads with a Gene Pulser (BIO RAD). Immediately following transfection cells were transferred to fresh LIT media and 16 hours later 100 µg/ml of G418 was added.

Results

Gene identification. Supernatants from the VIIA4 hybridoma, generated from the fusion of Sp2/0-Ag14 myeloma cells and spleen cells from mice immunized with an amastigote lysate as described in [19], recognize a ~90 kDa protein in western blots of amastigote lysate (refer to Chapter 2, Figure 1A). In addition, this antibody positively stains a subset of amastigotes analyzed by flow cytometry indicating surface expression (Chapter 2, Figure 1B). This monoclonal was then used to screen a *T. cruzi* amastigote cDNA library [19] resulting in the identification of a clone encoding a 2.7 kb open reading frame (ORF). BLAST analysis of the 902 amino acids encoded by this ORF revealed that amino acids 42-708 are 36% identical and 55% similar to the amino-terminal domain of the human β-3A subunit of the human AP-3 adaptor complex (NP_003655; Figure 3.1A)

with similar homology to the human β-3B adaptin (NP_004635) and the Drosophila β3 protein (AAF45950). Based on this homology we named this gene *Tcb3*. Tcβ3 is less homologous to the amino-terminal domains of the other human β adaptins, β1, β2 and β4 (NP_001118, NP_001273 and NP_006585), sharing 27% identity with β1 and β2 and 23% identity with β4.

Like other large adaptin molecules, the β adaptin molecules are composed of three domains, the amino-terminal "trunk" or "Adaptin N" domain, a hydrophilic "hinge" region and a carboxy-terminal "ear" domain (reviewed in [1]). The amino-terminal trunk region is the most conserved and is involved in interactions with the other adaptor subunits and with di-leucine based sorting signals in the cytoplasmic tails of target proteins [28-30]. The hinge domain of β adaptin molecules is characteristically hydrophilic, acidic and enriched in serine residues, many of which are potential phosphorylation sites [31, 32], but is variable both in sequence and length [1, 31]. Based on these characteristics the hinge region of Tcβ3 lies between amino acids 711 and 787, a hydrophilic region downstream of the N-terminal trunk domain that is enriched in acidic (26%) and serine (34%) residues (Figure 3.1B). In contrast to the hinge domains of human β1, β2, β3A, β3B and Drosophila β3, the hinge domain of Tcβ3 does not contain the clathrin-binding motif L (L, I)(D, E, N)(L, F)(D, E) [33-35]. However, Tcβ3 does contain an additional hinge-like region within the trunk domain that is extremely hydrophilic and rich in serine residues (51.28%). This unique 39 amino acid stretch (amino acids 297-335) terminates in a sequence, IDFLLD, which is reminiscent of the clathrin binding consensus motif. There is no significant homology between C-terminal domains of Tcβ3 and the other β3 proteins. This is not surprising as even the most

closely related human isoforms β3A and β3B are only 50% identical to one another in this region and share no homology to the C-termini of human β1 or β2. Also, this region is completely lacking in the yeast homologue and is lacking in the *T. brucei* β1 homologue [1, 31].

Localization. To further investigate the localization of Tcβ3, epimastigote forms of *T. cruzi* were stained with VIIA4 followed by a FITC-conjugated goat F(ab')$_2$ anti-mouse IgG (Figure 3.2C). As negative and positive controls respectively, cells were also stained with OKT4, a monoclonal antibody that recognizes human helper T-cells, or chronic mouse serum (Figure 3.2A and 3.2B respectively) followed by staining with the same secondary antibody. The immunofluorescence staining with VIIA4 revealed a distinct punctate pattern suggestive of vesicular association throughout the parasite but not co-localizing with nuclear or mitochondrial DNA (visualized by DAPI staining) suggesting exclusion from the corresponding organelles. The localization of endogenous Tcβ3 is very similar to the localization of other B3 proteins [4, 32, 36] and supports the hypothesis that in addition to being homologous in sequence Tcβ3 is a functional homologue.

To further characterize the localization of this protein we attempted to overexpress the full-length *Tcb3* as well as two truncated versions fused N-terminally to green fluorescent protein (GFP). These constructs were cloned into pRIBOTEX, an effective expression vector for both transient and stable protein expression in *T. cruzi* [21]. Epimastigotes were transfected with a control plasmid containing GFP alone (pRT-*GFP*) or the *Tcb3-GFP* fusion constructs containing the entire open reading frame (pRT-

2.7*Tcb3-GFP*), the N-terminal 834 bp or 1122 bp of *Tcb3* (pRT-0.8*Tcb3-GFP*, pRT-1.1*Tcb3-GFP* respectively). These truncations were selected because they flank the unique serine rich hydrophilic stretch in the N-terminal trunk domain. Parasites transfected with pRT-*GFP* alone displayed diffuse fluorescence distributed evenly throughout the cytoplasm but excluded from the nucleus (Figure 3.3A). In contrast, as shown in Figure 3.3B, the distribution of GFP was dramatically altered when expressed in fusion with the first 834 bp of *Tcb3* resulting in a punctate/vesicular-like pattern of fluorescence similar to the pattern exhibited by endogenous Tcβ3. This result indicates that the N-terminal 834 bp of *Tcb3* is sufficient to direct vesicular localization. GFP localization attained by fusion with the N-terminal 834 bp could not be compared to localization directed by additional *Tcb3* sequence because transient transfection with the 1kb and the full-length fusion did not lead to detectable GFP expression.

In order to address whether or not the lack of detectable GFP expression in parasites transfected with pRT-1.1*Tcb3-GFP* or pRT-2.7*Tcb3-GFP* was a consequence of GFP fusion, the same constructs were generated with a C-terminal myc epitope tag instead of GFP (pRT-0.8*Tcb3-myc*, pRT-1.1*Tcb3-myc*, pRT-2.7*Tcb3-myc)*. Parasites transfected with the *Tcb3-myc* constructs were stained with an anti-myc monoclonal antibody and examined by indirect immunofluorescence microscopy. The parasites transfected with the 0.8 kb *Tcb3-myc* construct exhibited the same localization pattern seen with the 0.8 kb *Tcb3-GFP* fusion (Figure 3.3C), again indicating that this sequence is sufficient to direct punctate localization, but transient transfection with the 1.0 kb and the 2.8 kb *Tcb3-myc* constructs did not result in detectable expression. Although GFP and myc could be detected in parasites transiently transfected with pRT-0.8*Tcb3-GFP*

and pRT-0.8*Tcb3-myc* respectively, this expression was not maintained after drug selection for stable transfectants.

Function. The results obtained with the GFP and myc expression constructs suggest that the Tcβ3 fusion proteins are acting as dominant negative mutants and that proper regulation of Tcβ3 is important for parasite survival. To further address this question, we attempted to knock out *Tcb3* by homologous recombination with constructs containing either the neomycin or hygromycin resistance genes flanked by sequence up and downstream of the *Tcb3* open reading frame. The fact that we were unable to recover drug-resistant parasites after transfection with either construct supports the hypothesis that Tcβ3 is essential to parasite survival (data not shown).

As an alternative to gene-knock out by homologous recombination the use of regulated RNA interference (RNAi) was explored. RNAi has been utilized successfully in *T. brucei* to inactivate gene function [24, 37-40] and our lab has preliminary data that RNAi functions in *T. cruzi*. However in *T. cruzi* we lacked high efficiency transformation and the ability to inducibly regulate expression of dsRNA in a wide variety of parasite strains, both critical components to the successful use of RNAi in *T. brucei* to study genes that are essential for parasite survival. In an attempt to circumvent this problem we modified the pZJM plasmid [24], capable of generating dsRNA from *T. brucei* alpha tubulin in a tetracycline regulated manner, by replacing its drug resistance marker gene with the enhanced GFP-puromycin chimeric gene from pEGFP-puro [25](Figure 3.4A). The resulting plasmid, pZJM.Ep, retains the non-regulated T7 promoter upstream of the pEGFP-puro gene. Replacing the drug resistance gene with the

EGFP-puro chimera allows for the identification and sorting of transfected parasites expressing GFP within hours of transfection, these sorted transfectants can then be monitored for the potential development of unique phenotypes (including lethality) during the 2-3 week period of drug selection. Transfection of *T. cruzi* expressing the T7 polymerase with pZJM.Ep containing a portion of *T. cruzi* alpha tubulin gene, *gp-72* (a protein critical for flagellar adhesion, [41]) or *Tcb3*, all genes whose loss would be predicted to cause a distinct phenotype, resulted in GFP positive parasites that were sorted and cultured in the presence of puromycin. However in all cases parasites in these cultures maintained GFP expression but failed to develop a distinctive phenotype either in growth or morphology.

To confirm that modifying pZJM by the replacement of the phleomycin resistance gene with EGFP-puro^r did not alter the ability to generate RNAi-induced phenotypes, we transfected procyclic *T. brucei* 29-13 cells, which contain integrated copies of T7 RNA polymerase and the tetracycline repressor, with pZJM.Ep. The pZJM.Ep construct contains 650 bp of the *T. brucei* α-tubulin gene and upon induction should result in the previously defined FAT phenotype [24]. As shown in Figure 3.4B, GFP positive FAT cells were detected 16 hours after induction with tetracycline, 32 hours post-transfection with pZJM.Ep. These results indicate pZJM.Ep is capable of inducing RNAi associated phenotypes in *T. brucei*.

Since further work is needed to establish RNAi in *T. cruzi*, we chose to utilize the well-established inducible RNAi system in *T. brucei* to assess the function of β3 adaptin-like proteins in these organisms. To identify the *T. brucei* β3 adaptin homologue, primers were designed based on highly conserved regions shared between Tcβ3 and other

β3 adaptins amino acids 86-250; (Figure 3.5A). These primers PCR amplified a 492 nt product from procyclic *T. brucei* cDNA that is 68% identical to the corresponding *Tcb3* nucleotide sequence and 73% identical, 83% similar at the amino acid level. The entire gene sequence is not necessary for RNAi and thus we proceeded with cloning the 492 nt PCR product into pZJM.Ep and the resulting construct, pZJM.Ep-*Tbb3*, was transfected into procyclic *T. brucei* cells as described below. BLAST (Basic Local Alignment Search Tool) analysis of the 492 nts PCR product against the GSS (genome survey sequence) database maintained by the National Center for Biotechnology Information (NCBI) identified a *T. brucei* sheared genomic DNA clone (AL461686) that was 99% identical to the C-terminal 300 bps of the PCR product. This genomic clone extended the C-terminal sequence by an additional 373 nts. Additional 5'end sequence was obtained by PCR amplification of procyclic *T. brucei* cDNA using a primer based on the *T. brucei* splice leader sequence and a reverse primer based on the 5' end 492 nt PCR amplified product described above. Collectively, this 5' sequence (334 nt), the initial 492 nt PCR amplified product and the sequence obtained from the GSS clone comprised a 1120 bp open reading frame that is 65.5% identical to the N-terminal *Tcb3* nucleotide sequence and 73.9% identical at the amino acid level (Figure 3.5B). Blast analysis of the 373 amino acids encoded by the *T. brucei* open reading frame revealed that it, like Tcβ3, shared the highest homology with the N-terminal trunk domains of the human β3B and β3A adaptins, 38% and 37% identical and 61% and 58% similar respectively. Based on these findings this sequence will be referred to as the N-terminal sequence of Tbβ3 or Tbβ3-N.

To determine whether or not RNAi of Tbβ3 induces a detectable phenotype in *T. brucei* procyclics, a fragment of the *Tbb3-N* sequence was cloned into pZJM.Ep, replacing the *T. brucei* α-tubulin gene fragment as described in Materials and Methods. A negative control plasmid, pZJM.Ep-*no2T7*, was generated by removing the dual T7 cassette by digesting pZJM.Ep with Kpn I and BamH I and subsequent religation. This plasmid retained the ability to express the EGFP-puro gene in the presence of T7 polymerase (data not shown). *T. brucei* 29-13 procyclic cells were transfected with either pZJM.Ep-*Tbb3* or pZJM.Ep-*no2T7* selected for 3 weeks in the presence of 10 μg/ml puromycin to select for stable transfectants and then induced to express the dsRNA by the addition of 1 μg/ml tetracycline. A reduction in growth-rate became evident 5 days after induction of the pZJM.Ep-*Tbb3* transfected cells and the growth of these cells was reduced by 140 fold by day 12 of induction as compared to uninduced pZJM.Ep-*Tbb3* transfected cells and to the cells transfected with pZJM.Ep-*no2t7* regardless of induction (Figure 3.6).

Fluorescence and light microscopy revealed a distinct phenotype in many of the pZJM.Ep-*Tbb3* transfected cells that became evident after five days of induction (Figure 3.7B). These cells exhibit at least one detached flagellum and appear to have a block in cytokinesis as indicated by an abnormal number of nuclei and/ or kinetoplasts, visualized by DAPI staining. Non-induced cells transfected with the same construct appeared morphologically normal at this time point (Figure 3.7A). During the normal *T. brucei* cell cycle the kinetoplast replicates and segregates before mitosis occurs, thus the progression is from a single kinetoplast and single nucleus, 1K1N, to 2K1N, 2K2N and finally cytokinesis occurs resulting in 2 new 1K1N daughter cells. The replication and

segregation of the kinetoplasts is linked to the formation of the new flagellum. As illustrated in Figure 3.7B, expression of dsRNA from *Tbb3* does not inhibit the formation of a new flagellum or the replication of either nuclear or kinetoplast DNA, the cells shown in the left two panels are at least 4N and the cells in the two panels on the right are at least 3K, situations that would not occur if cytokinesis had taken place.

Discussion

The cytoskeletal structure of trypanosomes presents unique challenges to the vesicular trafficking machinery in these organisms. An array of highly organized and tightly spaced microtubules lies directly beneath the plasma membrane preventing vesicular fusion at most locations. This subpellicular corset is absent underneath the flagellar pocket, a region formed by the invagination of the plasma membrane and the emergence of the flagellum, and it is through the membrane of the flagellar pocket that all endo and exocytosis occurs (reviewed in [7]). The membrane of the flagellar pocket consists of only 0.4-3% of the total plasma membrane [17, 42] yet supports a remarkably high level of endocytosis. For example the rate of endocytosis in *T. brucei* bloodstream forms is one of highest ever reported, turning over the equivalent of the surface area of the flagellar pocket membrane every 1-2 minutes [43]. Consequently proteins involved in vesicular trafficking to and from the flagellar pocket region would be predicted to play an essential role in parasite survival.

In the course of screening a *T. cruzi* amastigote cDNA library for potential vaccine candidates, a gene was identified that showed extensive homology to the amino-terminal domain of the human β3A and β3B and the Drosophila β3 adaptin proteins and

was named Tcβ3. The β3 adaptins are one of the large subunits that compose the heterotetrameric AP-3 adaptor complex. Two forms of the AP-3 complex have been characterized in mice and humans, a ubiquitously expressed complex that is involved in vesicular trafficking from the TGN and /or endosomal compartments to lysosomal and lysosomal-like organelles and a neuronal-specific complex that is involved in the formation of a subset of synaptic vesicles from endosomal compartments (reviewed in [1, 44]).

Despite recent interest in understanding the potential unique modes of vesicle trafficking in trypanosomes, members of the adaptin family have not been well characterized in these organisms. For this reason and because of the unique constraints placed on vesicular trafficking in these organisms we decided to pursue the characterization of Tcβ3. Sequence analysis revealed that the Tcβ3 contained the three domains characteristic of other β adaptins, a highly conserved N-terminal trunk region, a hydrophilic hinge region enriched in serine residues, and a C terminal domain that shares very little homology with the other β adaptins. Immunofluorescent staining revealed that Tcβ3 protein exhibits a punctate/ vesicular pattern throughout the cell body of *T. cruzi* epimastigotes, similar to the observed localization of human and Drosophila AP-3 adaptins [3, 4, 36]. This localization pattern was confirmed by the punctate localization of GFP and myc in parasites transfected with constructs containing the N-terminal 0.834 kb of *Tcb3* fused with GFP or tagged with myc. The similarities in sequence domain organization and in localization patterns are consistent with the conclusion that Tcβ3 is a functional adaptin molecule.

The lack of detectable GFP and myc expression in parasites transfected with 1.1 kb and the 2.7 kb constructs and the inability to obtain stable transfectants expressing the 0.8 kb constructs suggests that these constructs were acting as dominant negative mutants. These results indicate that proper regulation of Tcβ3 expression is critical for parasite survival, a hypothesis that is supported by our failure to generate single or double knockouts of *Tcb3* by homologous recombination in *T. cruzi*.

We turned to RNAi as an alternative approach to characterize the function of Tcβ3. Assessing the function of an essential gene in *T. cruzi* by RNAi is complicated because an inducible expression system that is widely usable has not been developed in this organism. Expression of a lethal dsRNA most likely will lead to parasite death during the drug selection process. To circumvent this problem we created the dsRNA generating plasmid, pZJM.Ep, that allows the detection and purification of transfected parasites within hours of electroporation by fluorescence based cell sorting. This construct was accomplished by replacing the drug resistance marker in pZJM [24], a plasmid capable of generating dsRNA that has been extensively used in *T. brucei* [24, 39, 40], with a chimeric GFP/puromycin resistance gene [25]. This plasmid theoretically allows for the phenotypic analysis of parasites expressing potentially lethal RNAi constructs. Initial testing of this plasmid containing fragments of *T. cruzi* genes whose loss would be predicted to lead to a distinct phenotype, *α-tubulin*, *gp-72* (critical for flagellar adhesion) or *Tcb3*, resulted in fluorescent parasites that did not exhibit any noticeable morphological or growth phenotypes. However *T. brucei* procyclics transfected with pZJM.Ep exhibited the FAT phenotype associated with loss of α tubulin [45], confirming that transfection with this plasmid can generate dsRNA induced

phenotypes. At a minimum the failure to observe similar phenotypes in *T. cruzi* suggests that dsRNA-mediated interference is not as efficient in *T. cruzi* as it is in *T. brucei.* However the utility of RNAi in *T. cruzi* merits further investigation.

As we continue to pursue RNAi in *T. cruzi,* we decided to attempt to identify a *T. brucei* homologue of Tcβ3 and to analyze its function by inducible RNAi, a well-established technique in *T. brucei* [24, 37-40]. Using primers based on conserved sequences between Tcβ3 and other related β3 adaptins, corresponding to amino acids 86-250 of Tcβ3, a PCR product was amplified from procyclic *T. brucei* cDNA. This 492 nt PCR, additional 5' end sequence generated by PCR amplification using a splice leader primer and additional 3' sequence was obtained from a short GSS match were combined to yield a 1120 bp open reading frame that shared significant homology to *Tcb3* as well as the N-terminal domains of human and Drosophila β3 adaptin molecules. Confident that we had identified the N-terminus of the *T. brucei* β3 homologue, pZJM.Ep-*Tbb3,* containing the initial 494 nt PCR product, was transfected into procyclic *T. brucei* cells and dsRNA production was initiated using tetracycline. A reduction in growth was noticeable within five days of induction and led to a 140-fold decrease in cell density by day 12. Microscopic analysis revealed that many of the cells expressing the Tbβ3 dsRNA did not undergo cytokinesis leading to cells containing multiple kinetoplasts or nuclei than normally and in addition, most of these cells had at least one detached flagellum.

These data confirm that Tbβ3 plays an essential role in parasite survival but they do not reveal the specificities of its function. One could interpret both the flagellar detachment phenotype and the apparent block in cytokinesis as an indication that Tbβ3 is

directly involved in the trafficking of proteins that are critical for these functions. However these phenotypes could be a downstream effect of numerous other deficiencies. Since AP-3 has been demonstrated in many organisms to be involved in vesicular trafficking to lysosomes and lysosomal-like compartments (reviewed in [1, 44], a more direct evaluation of the effects of interfering with Tbβ3 function would be to observe the localization of a lysosomal marker whose localization would be predicted to be AP-3 dependent, like p67. p67 is a structural and functional homologue of the mammalian lysosome-associated membrane proteins (LAMPs) [46, 47]. In mammalian cells LAMP lysosomal targeting is strictly AP-3 dependant and is mediated by sorting signals in the cytoplasmic domain [29, 48]. Likewise the lysosomal targeting of p67 is dependent on its cytoplasmic domain as evidenced by the ability of this region to direct lysosomal localization of GFP and by the data showing that p67 is misrouted to the plasma membrane if the cytoplasmic domain is removed [47]. Therefore we are currently investigating the localization of p67 in parasites expressing dsRNA for Tbβ3. If our hypothesis that Tbβ3 is involved in trafficking of p67 to the lysosome is correct then p67 should be detected on the cell surface instead of the lysosome in parasites with a deficiency in Tbβ3.

Although the specific function of the *T. cruzi* and *T. brucei* β3-like molecules identified in this study are still being elucidated, the data presented here indicate that these molecules are essential in trypanosomes. Deficiencies in AP-3 adaptin molecules in yeast, Drosophila, mice and humans lead to defects associated with misrouting of lysosome or lysosomal-like proteins, however these molecules are not essential for the

survival of any of these organisms [1, 35, 49-53]. This suggests that trypanosomes are more dependent on functional AP-3 complexes.

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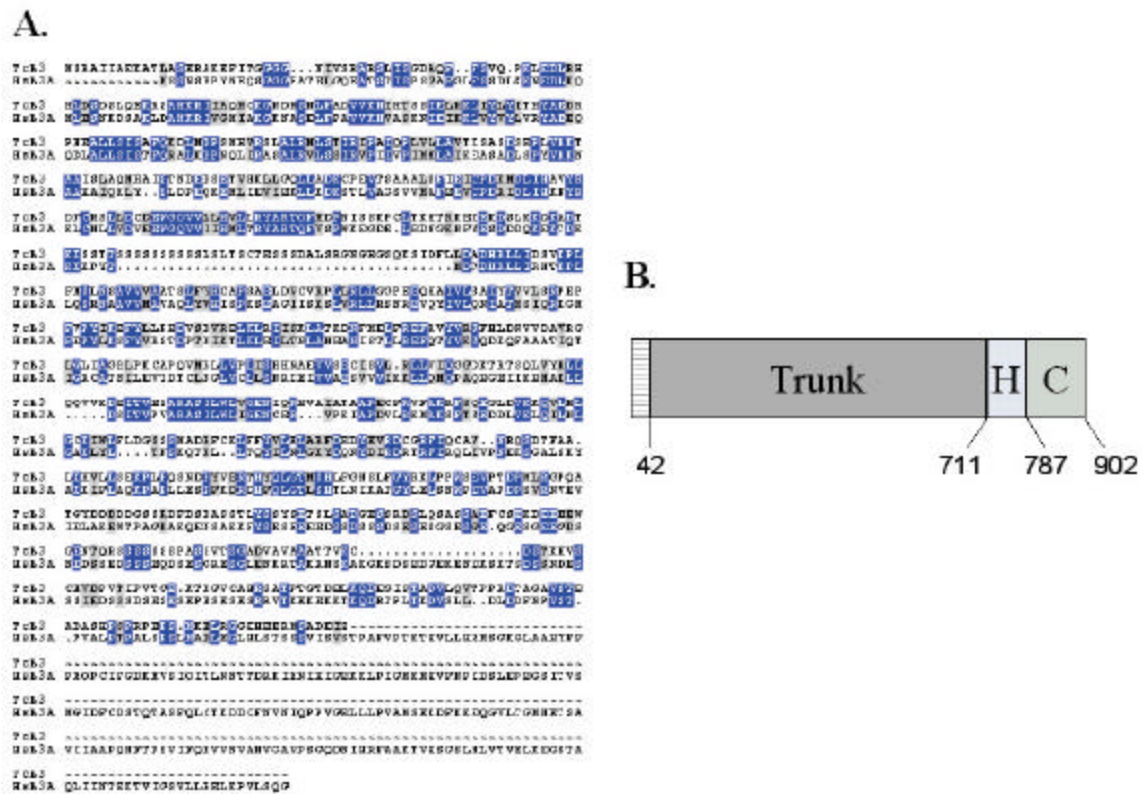


Figure 3.1. Tcb3 is homologous to b3 adaptins. A. Multiple sequence alignment comparing the amino acid sequence of Tcβ3 to the human β3A adaptin (NP_003655). Multiple sequence alignment was created by PILEUP and shaded using BOXSHADE. Identical residues are shaded in blue and similar residues in grey. B. Bar diagram showing the trunk, hinge (H), and C-terminal (C) domains of Tcβ3. Amino acid positions are noted.

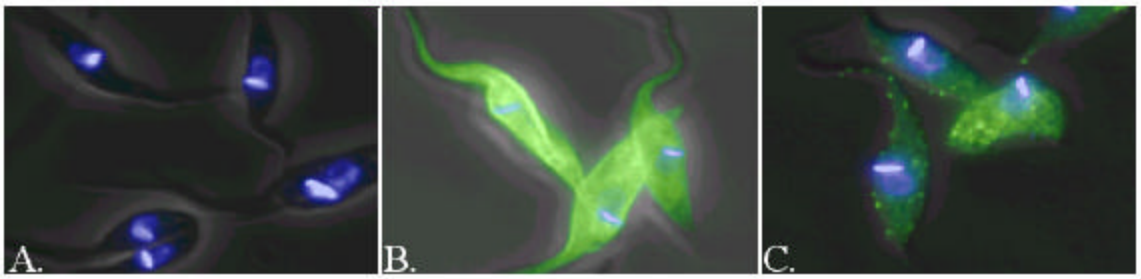


Figure 3.2. Endogenous Tcb3 is distributed in a distinct punctate pattern throughout the cell. *T. cruzi* epimastigotes were incubated with OKT4 (negative control antibody), chronic mouse serum (positive control) or VIIA4 (Tcβ3 specific antibody)(A, B and C respectively) and subsequently stained with a secondary FITC-labeled goat $F(ab')_2$ anti-mouse IgG. DAPI, staining the nuclear and kinetoplast DNA, represented in blue.

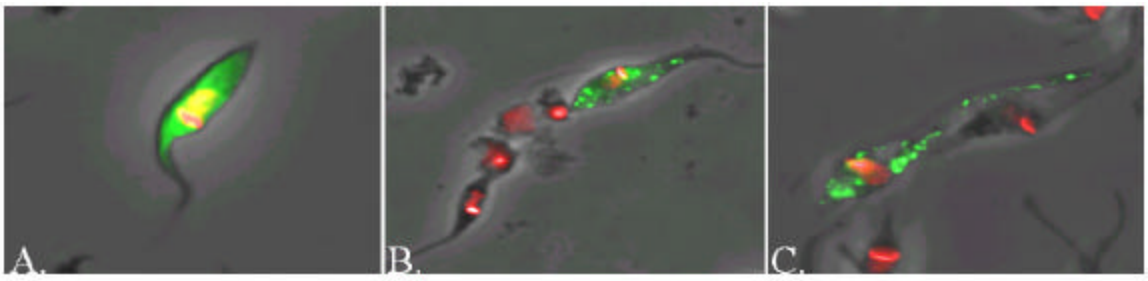
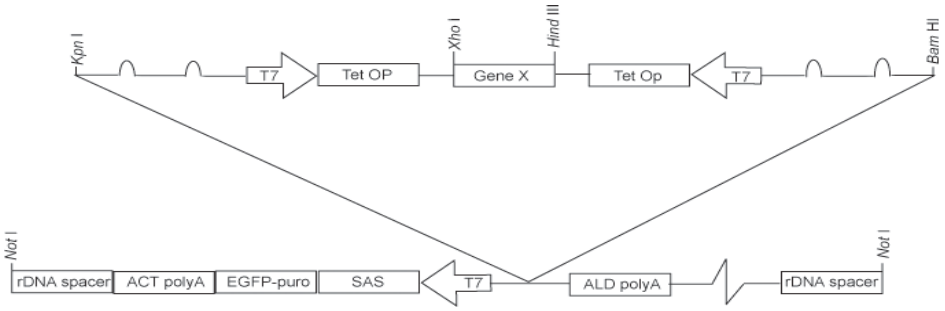


Figure 3.3. The N-terminal 834 bp of Tcb3 is capable of directing vesicular localization. Epimastigotes of *T. cruzi* were transfected with pRT-*GFP* (A), pRT-0.8*Tcb3-GFP* (B). 24 hours post-transfection cells were fixed, permeabilized and stained with DAPI, a DNA intercalating dye, shown in red. C. Cells transfected with pRT-0.8*Tcb3-myc* were fixed in methanol 24 hours post-transfection and stained with an anti-myc antibody followed by staining with a FITC-labeled secondary antibody and subsequent DAPI staining, shown in red.

A.



B.

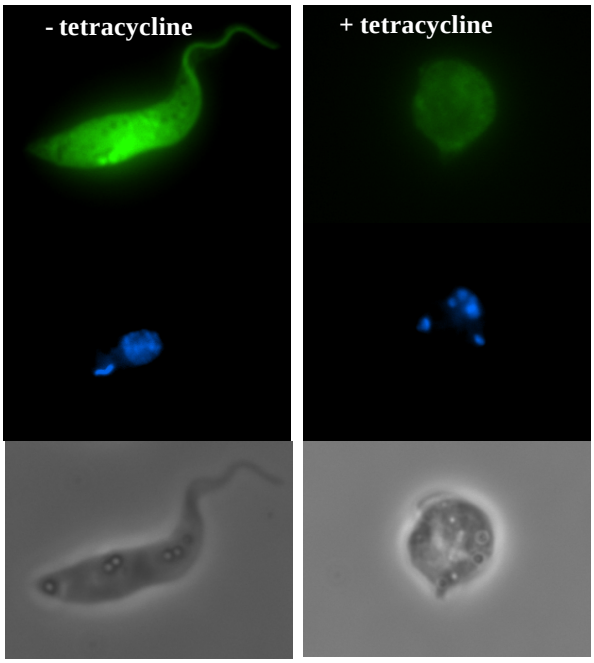


Figure 3.4. Modified pZJM vector, pZJM.Ep (A.), induces the FAT phenotype in *T. brucei* procyclics (B.). A. pZJM.Ep was generated by replacing the phleomycin drug resistance marker of pZJM with a chimeric enhanced GFP-puromycin resistance gene, EGFP-puro. B. *T. brucei* 29-13 cells were transfected with 20 µg of Not I linearized pZJM.Ep, 16 hours post-transfection cells were cultured in the absense (left panel) or presence of 1 µg/ ml tetracycline (right panel). 16 hours post-induction cells were fixed permeabilized and stained with DAPI (shown in blue).

A.

Gene	Forward primer	Reverse primer
TcB3	CCGATGTAGTGAAGAAC (nts 257-273)	GAGTGGGGGCAGGTGGT (nts 733-749)
Hs B3A	.T.C...T.....T (nts 242-258)	.....T.. (nts 712-728)
Hs B3B	...CG..G..... (nts 227-243)	.....C..... (nts 697-713)
Dm B3	.....G.CGC...C.T (nts 119-135)	..A.....T.....A. (nts 715-731)

B.

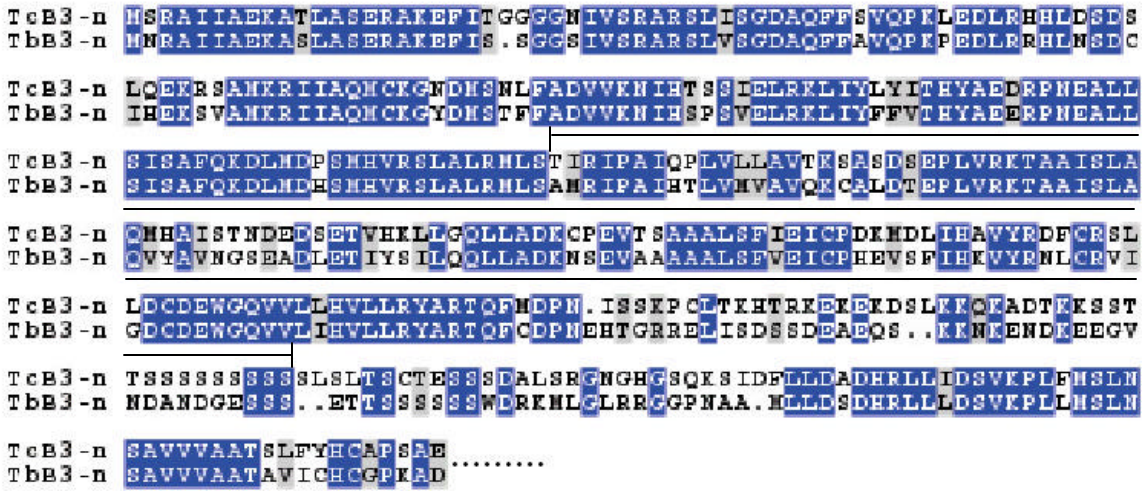


Figure 3.5. Identification of a b **3 homolog from *T. brucei*.** A. Primers used to PCR amplify the β3 homologue from *T. brucei* were based on the Tcβ3 sequence in areas that were conserved at the amino acid and to some degree the nucleotide level with the human β3A and β3B and the Drosophila β3 adaptins. Mismatched nucleotides are indicated by letter and identical matches are represented by a period. B. Multiple sequence alignment comparing the N-terminus of Tcβ3 to the 373 amino acid fragment of the newly identified *T. brucei* β3 homologue. These sequences are 73.9% identical on the amino acid level. Multiple sequence alignment was created by PILEUP and shaded using BOXSHADE. Identical residues are shaded in blue and similar residues in grey. Sequence corresponding to the fragment of Tbβ3 used for RNAi is underlined.

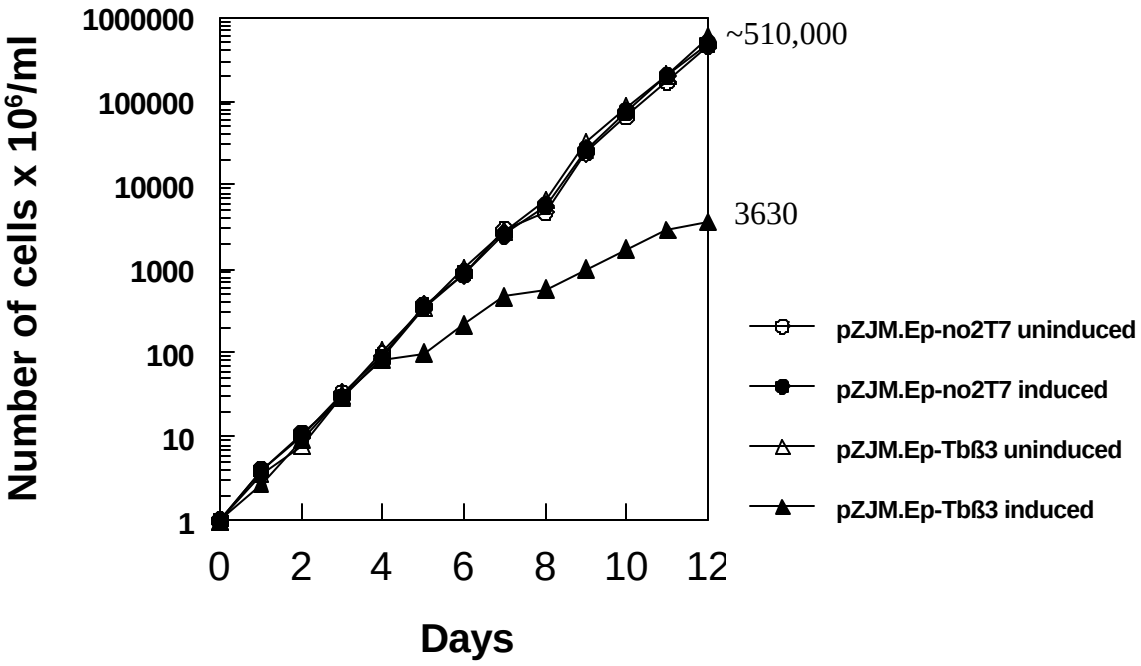


Figure 3.6. Induction of dsRNA production of Tbb **3 in *T. brucei* procyclics leads to a reduction in growth rate.** *T. brucei* 29-13 procyclics were transfected with 20 µg of (Not I linearized) pZJM.Ep-*no2T7* or pZJM.Ep-*Tbb3* and selected for 3 weeks in the presence of 10 µg/ ml of puromycin. The cells were then cultured at 10^6 cells/ml in either the absence or presence of 1 µg/ ml tetracycline. Cells were counted every 24 hours. When cells exceeded 5 x 10^6/ ml they were diluted to 10^6/ ml and counts were adjusted by the respective dilution factors.

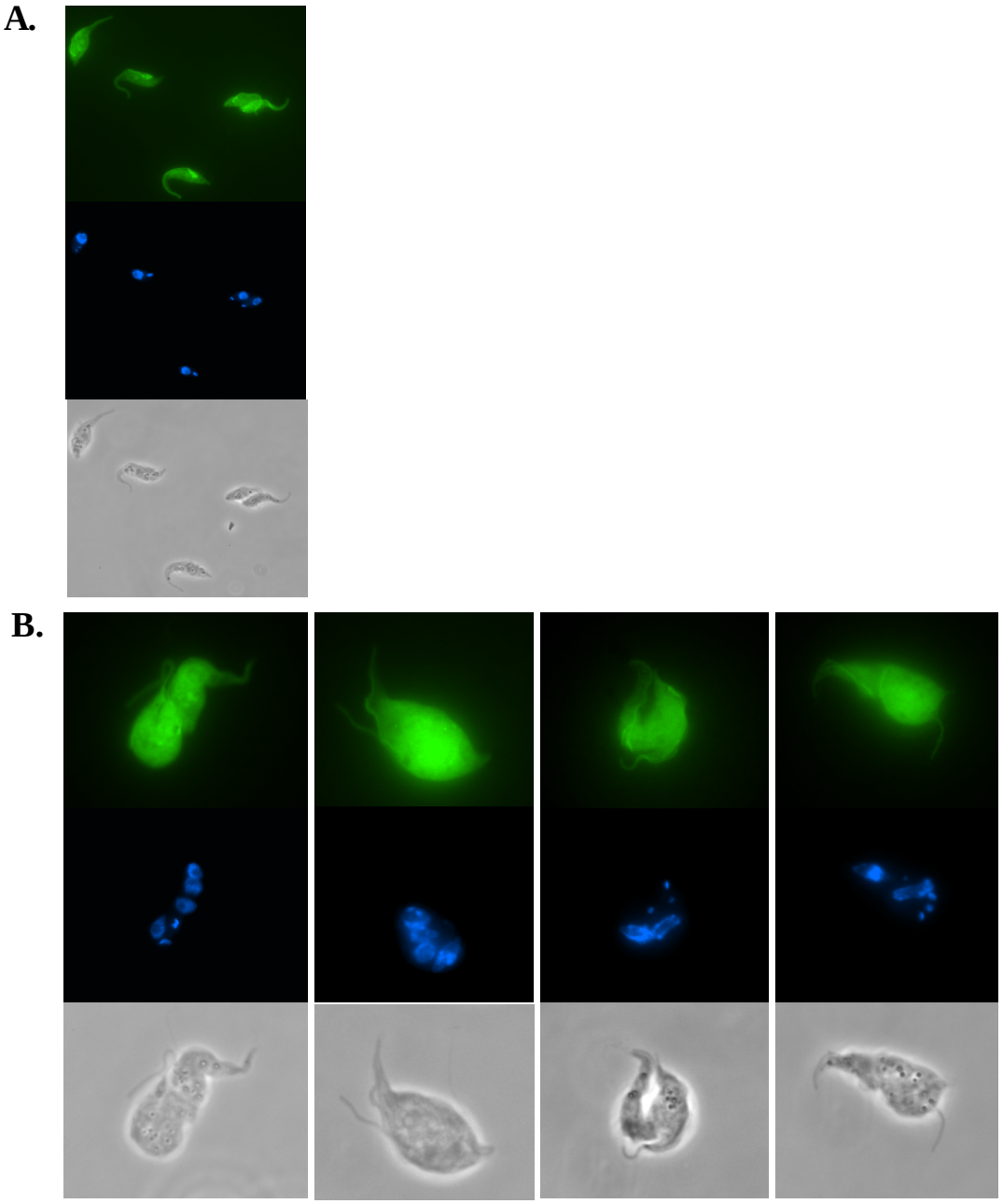


Figure 3.7. Expression of dsRNA from Tbb 3 leads to abnormal morphology.

pZJM.Ep-Tbβ3 transfected *T. brucei* were grown in the absence (A) or presence (B) of 1 μg/ml tetracycline and imaged day 5 post-induction. Cytokinesis appears to be blocked in these cells leading to the accumulation of nuclei and kinetoplasts. In addition, the newly forming flagella are detached.

CHAPTER 4

CONCLUSIONS

The data presented in this dissertation demonstrate that immunization with LYT1 or a pool of trans-sialidase (TS) genes protects mice from a normally lethal challenge of *T. cruzi*. These findings expand the diversity of genes that have been assessed as genetic vaccines for *T. cruzi* and validates the use of pools of genes as potential vaccines. In addition, the search for vaccine candidates resulted in the identification of a novel *T. cruzi* gene, Tcβ3, that is homologous to the human and Drosophila β3 adaptin proteins which are subunits of the AP-3 adaptor protein complex [1]. Functional characterization in higher eukaryotes has demonstrated that these adaptor complexes are essential for the recruitment of both vesicle cargo and coat proteins (reviewed in [1-3]). Tcβ3 was shown to be localized in a vesicular pattern throughout the cell and was able to direct vesicular localization of GFP and myc. Regulated RNAi was used to study the function of a β3 homologue from *T. brucei*, Tbβ3. RNAi of Tbβ3 leads to a significant growth defect, loss of flagellar adhesion and a block in cytokinesis, indicating that this gene is essential. Collectively, these data open new lines of investigation for future vaccination and cell biological studies.

The efficacy of vaccinating against *T. cruzi* with either single genes, Tcβ3, FCaBP and LYT1, or with gene pools containing members of either the TS or the mucin multi-gene family was evaluated. Immunizing with either LYT1 or the pool of TS genes protected mice from *T. cruzi* induced mortality, whereas immunization with the FCaBP

or Tcβ3 did not. We predicted that LYT1 would be an effective vaccine candidate because of the evidence suggesting that it is secreted from the parasite during host cell infection [4]. In the cytoplasm LYT1 would have access to the host cell MHC class I processing pathway and thus exposure to $CD8^+$ T cells, which are critical for control of *T. cruzi* infection [5-7]. Although peptides from all three genes were targets of $CD8^+$ T cells from chronically infected mice only immunization with LYT1 conferred protection. These data indicate that not all $CD8^+$ T cell targets are effective vaccines. Out of the three individual genes tested LYT1 is the only true secreted protein, neither FCaBP or Tcβ3 contain signal sequences, possibly indicating that proteins actively secreted from the intracellular stage of the parasite may be more effective as vaccines. These results suggest that as more genome sequence information for *T. cruzi* becomes available it might be useful to focus the search for genetic vaccines on genes that encode potential secretory proteins.

As this study was ongoing the first reports utilizing genetic vaccination in *T. cruzi* were published [8-12]. The majority of the genes that have been tested to date are members of the TS gene superfamily and more specifically all but one of them are members of the TS-like subfamily. The TS supergene family can be divided into two main categories, the TS family consisting of approximately 140 genes, approximately half of which are enzymatically active, and the TS-like family consisting of over 700 genes that are not enzymatically active [13]. We initially focused on identifying and characterizing non-TS genes because of the suggestion that the conservation of related epitopes in different members of the TS family might function as altered peptide ligand antagonists to subvert the immune response [14, 15]. Therefore immunizing with these

genes may not elicit the optimal response. However because of the emergence of data validating TS supergene family members as effective vaccine candidates we chose to evaluate additional TS family members. We found that immunizing with a pool of genes from the TS subfamily induced protection from a normally lethal challenge of *T. cruzi*. Interestingly, immunizing with a pool of mucin genes did not. The mucin family is the other large *T. cruzi* gene family that also encodes GPI-anchored surface proteins that are well-characterized targets of immune responses but had not been previously evaluated as vaccine candidates [13]. Although mucins like TSs are highly expressed surface proteins they differ from the TS molecules in that they are heavily glycosylated [16]. Recent evidence suggests that mucins are capable of causing substantial inhibition of lymphocytes that is dependent on their glycosylation [17]. This data may explain why immunization with a pool of mucin genes did not protect against *T. cruzi* infection.

The results of this study indicate that LYT1 and pools of TS genes are effective as vaccines for *T. cruzi*, expanding the collection of genes that are known to induce protection against *T. cruzi* and establishing that pools of genes can be used effectively. Immunizing with pools of genes increases the diversity of the vaccine that is critical for protecting a diverse human population against infection with a diverse population of parasite strains. The TS genes assessed in this study are distinct from the TS superfamily genes that have been previously tested the majority of which are members of the TS-like subfamily not the TS subfamily. It would be interesting to investigate whether immunizing with an even more complex mixture of TS genes, including members of the TS-like subfamily as well as TS genes, in addition to LYT1 increases the degree of protection.

Although Tcβ3 was not an effective vaccine candidate, its potential role in protein trafficking in trypanosomes, based on its sequence homology to β3 adaptins, inspired us to investigate its function. Very little is known about the molecules involved in vesicular trafficking in trypanosomes, organisms in which all of the endo- and exocytosis is limited to the very small region of the plasma membrane surrounding the flagellar pocket [18, 19]. The possibility that these structural constraints may necessitate novel mechanisms of protein trafficking further justifies characterization of proteins that may be involved in these pathways.

Localization studies of both endogenous and exogenously expressed Tcβ3 in *T. cruzi* revealed a vesicular/punctate pattern further supporting the conclusion that it is a functional adaptin molecule. Because we were unable to generate Tcβ3 loss of function parasites in *T. cruzi*, possibly indicting that it is an essential protein, we used its sequence to identify the N-terminus of the β3 homologue from *T. brucei*, Tbβ3. One advantage of working with *T. brucei* is the well-developed tetracycline regulated expression system that has been established in this organism [20], something that is not fully developed in *T. cruzi*. In addition RNA interference (RNAi) has been utilized extensively in *T. brucei* and is very powerful tool in combination with a regulated expression system [21-25]. The induction of dsRNA for Tbβ3 in *T. brucei* procyclic cells led to a significant growth defect, loss of flagellar adhesion and an apparent block in cytokinesis.

Although these data signify that the β3 adaptin-like molecule is essential in *T. brucei*, they do not give a clear indication of the function of Tbβ3. One interpretation of the observed flagellar detachment phenotype is that Tbβ3 is involved in trafficking proteins required for flagellar adhesion, yet this could be secondary to another defect(s).

Similarly, the potential causes for inhibition of cytokinesis are countless. A more direct assessment of β3 function would be to determine the localization of a protein whose trafficking would be predicted to be AP-3 dependant in parasites expressing dsRNA for Tbβ3. As an extension of the study presented here, we are currently investigating the localization of one such *T. brucei* protein, p67 [26], a structural and functional homologue of the mammalian lysosome-associated membrane proteins (LAMPs). In mammalian cells LAMP lysosomal targeting is strictly AP-3 dependant and is mediated by sorting signals in the cytoplasmic domain [27, 28]. Similarly, the lysosomal targeting of p67 is directed by its cytoplasmic domain (most likely by its two di-leucine motifs); the cytoplasmic domain of p67 directs GFP to the lysosome and if this region is truncated then p67 is routed the surface of the parasite and in [29]. Therefore we hypothesize that the lysosomal targeting of p67, like mammalian LAMPs, is mediated by AP-3 and predict that p67 will be misrouted to the plasma membrane in parasites expressing dsRNA for Tbβ3. We will assess the localization of p67 in clonal procyclic lines expressing dsRNA for Tbβ3 by immunofluorescence with a p67 mAb. Although defects in the AP-3 adaptins cause distinct phenotypes, characterized by misrouting of lysosomal or lysosomal-like proteins, they have not been shown to be lethal in yeast, Drosophila, mice or humans (reviewed in [1]). This suggests trypanosomes are more dependent on functional AP-3 complexes.

In conclusion, in this study we have identified genetic vaccine candidates, LYT1 and a subset of TS genes, that protect mice from *T. cruzi* induced mortality. This finding is significant because it substantially expands the diversity of genes that have been shown to be effective vaccines for *T. cruzi*. An effective field vaccine will have to protect a

diverse human population against a diverse parasite population and therefore most likely consist of many antigens. In addition we identified a novel β3 adaptin-like gene from *T. cruzi* and *T. brucei* and have shown that it is essential for parasite survival. The results presented here contribute to a better understanding of the immunity against as well of the cell biology of *Trypanosoma cruzi*.

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