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BIOCHEMICAL INVESTIGATION INTO INITIATION OF FATTY ACID SYNTHESIS IN THE AFRICAN TRYPANOSOMES

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BIOCHEMICAL INVESTIGATION INTO INITIATION OF FATTY ACID
SYNTHESIS IN THE AFRICAN TRYPANOSOMES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biochemistry and Molecular Biology

by
Sunayan S. Ray
August 2013

Accepted by:
Dr. Kimberly S. Paul, Committee Chair
Dr. James C. Morris
Dr. Kerry Smith
Dr. Michael Sehorn

ABSTRACT

My doctoral studies focused on studying FA metabolism in the deadly protozoan parasite *T. brucei*. In my dissertation, I addressed various aspects of the regulation of TbACC, which catalyzes the first committed step in FA synthesis. In the second chapter, I hypothesized that TbACC is regulated in response to environmental lipids. I examined changes in TbACC RNA, protein abundance, and enzymatic activity in response to different levels of environmental lipids in both BF and PF cells. I also delineated the mechanisms by which TbACC expression and activity is regulated by phosphorylation in response to altered lipid environments. In the third chapter, which has been published, we tested the effects of a compound in green tea extract known as epigallocatechin gallate (EGCG), on growth of *T. brucei*, and on TbACC activity and phosphorylation. EGCG is a known inducer of AMPK, which phosphorylates ACC in other organisms. In the fourth chapter, I demonstrated that TbACC in PF is also regulated by various allosteric regulators. I also showed that TbACC might form oligomers. Together these studies have given an insight on the ability of *T. brucei* to regulate its FA synthesis and the role this pathway may play in the survival of this deadly parasite in its hosts. This knowledge may be exploited in the future to find a better cure for Trypanosomiasis.

DEDICATION

My dissertation is dedicated to my mom, Sangeeta S. Ray, who gave me strength through her prayers, my brother, Nipun Ray, who gave me all the encouragement and my dad, Dr Maheshwar S. Ray, who gave me inspiration.

Above all I will dedicate this to my only Grandma Sarojini Pal who has always been a source of joy and optimism.

ACKNOWLEDGMENTS

Words are not enough to express my gratitude to all those persons who have helped and guided me throughout my life.

I would like to gratefully acknowledge Dr Kimberly S Paul for giving me the opportunity to work as a graduate student in her lab. Knowledge acquired during the duration of my studies in the Paul lab will definitely help me open new gateways and avenues to my journey into the quest of science.

I would also express my heartiest gratitude to my committee members for providing me with precise and apt knowledge pertaining to my project. Without their assistance none of this work would have been possible.

I am indebted to all Paul lab members for providing me with an excellent work environment during my stay in the lab.

I am obliged to thank all my teachers, family back in India, and family here away from home. Without their support, encouragement, benevolence, and blessing I would not have been in Clemson.

And finally, but immensely, I would like to express my gratitude to all my lovely friends in CLEMSON who taught me to bleed orange and made me feel at home. These are the people who directly or indirectly helped me by keeping my spirits up and my mind in working order.

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CHAPTER ONE LITERATURE REVIEW

INTRODUCTION

Trypanosoma brucei, an early branching protozoan belonging to the family Trypanosomatidae is a 15-30 µm long, highly motile organism powered by a single flagellum. At the base of the flagellum is the kinetoplast, which is the highly condensed genome of the single tubular mitochondrion and marks *T. brucei* as a member of the class of Kinetoplastida, which contains both free-living and parasitic species. *T. brucei* species causes fatal disease in humans and animals and are transmitted by an insect vector, the tsetse fly (*Glossina* species).

Trypanosomiasis affects people and animals living in the ~6.2 million square miles of sub-Saharan Africa (FAO, Food and Agricultural Organization of the United Nations, 2007). The World Health Organization (WHO) indicates that between 1998 and 2004 there were an estimated 50,000 – 70,000 cases annually of human African trypanosomiasis (HAT) (WHO, 2010). More recently sustained control efforts have reduced the number of reported HAT cases, with 6,743 and 7,197 cases reported in 2011 and 2012, respectively. However, HAT remains a major public health concern in 36 countries (Simmaro *et al.*, 2009), particularly in some regions (Democratic Republic of Congo, Angola, and South Sudan), where the infection rate is especially high, exceeding malaria or HIV/AIDS as a cause of death (WHO, 2010).

Three different *T. brucei* sub-species affect sub-Saharan Africa: *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*. *T. b. brucei* causes disease in domestic and wild animals but not humans. *T. b. brucei*'s inability to infect humans can be attributed to its susceptibility to human trypanolytic factor (TLF), which is a type of high density lipoprotein particle in human serum. Endocytosis of TLF by *T. b. brucei* causes lysis of the parasites (Wheeler, 2010). Cattle and other mammals lack TLF in their serum, which makes them susceptible to infection by *T. b. brucei*. However, *T. b. rhodesiense* and *T. b. gambiense* are resistant to TLF and therefore can successfully infect humans. *T. b. gambiense* causes endemic disease in central and west Africa, while *T. b. rhodesiense* affects east and south Africa (Brun *et al.*, 2010) (Fig. 1.1). The Nile Rift Valley is responsible for the strict geographical distribution that physically separates the insect vectors bearing these two human infective strains. However, in northwest Uganda these strains do overlap due to the occurrence of both strain-bearing vectors.

AFRICAN TRYPANOSOMIASIS

African Sleeping Sickness

HAT, also commonly known as African sleeping sickness, differs depending on which sub-species causes infection. *T. b. gambiense* causes a chronic infection

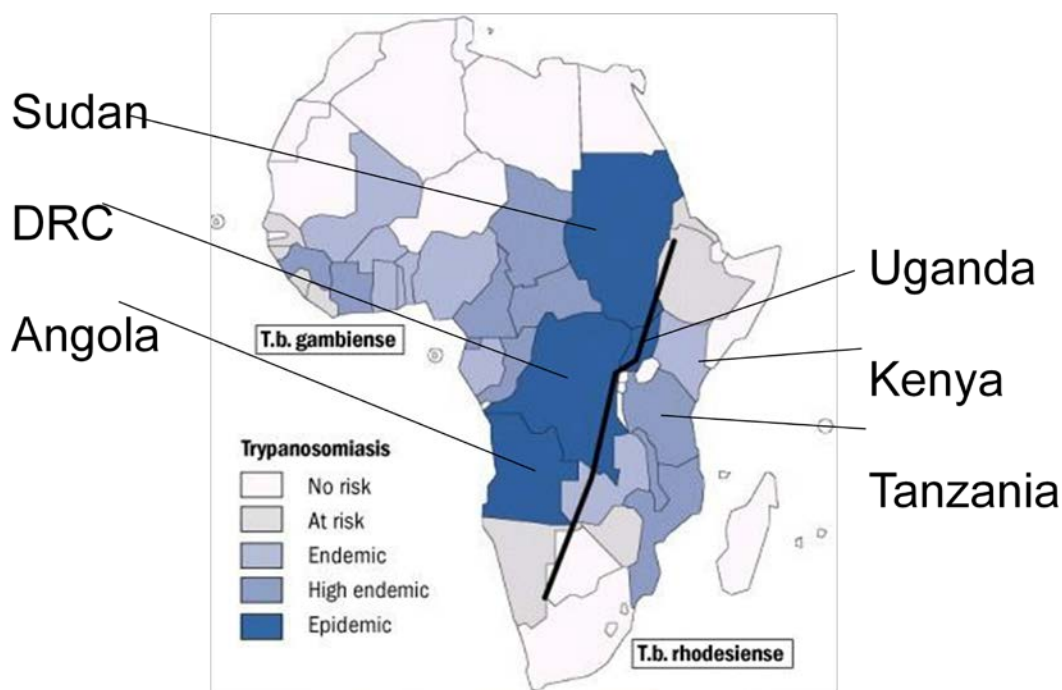


Figure 1.1: Geographic distribution of *T. brucei*. *T. b. gambiense* causes endemic disease in central and west Africa, whereas *T. b. rhodesiense* affects east and south Africa. Both strains are geographically separated by the physical separation of their corresponding insect vectors by the Great Rift Valley (WHO, 1999).

with death occurring after ~3 years, while *T. b. rhodesiense* causes an acute infection resulting in death in 2-3 months (Brun *et al.*, 2010). There are reports that demonstrate in some untreated patients that *T. b. gambiense* infection is not always fatal. However, very little is known about the frequency of self-cure or latent asymptomatic infections (Jamonneau *et al.*, 2012).

There are two distinct clinical stages of HAT: the haemolympathic stage (stage I) and the meningo-encephalitic stage (stage II). In stage I, the parasite is

limited to the bloodstream and lymph nodes. This results in non-specific symptoms such as intermittent fever, headache, lymphadenopathy (swollen lymph nodes), pruritus (itching), joint pains, and to lesser extent hepatosplenomegaly (enlarged liver or spleen). If infection is not treated in stage I, then the disease progresses to stage II after several weeks, months, or even years. In stage II, the parasite crosses the blood-brain barrier and invades the cerebrospinal fluid. Stage II symptoms include dramatic neurological and psychiatric symptoms, including sleep disturbances, tremor, fasciculation (involuntary muscle contraction and relaxation), general motor weakness, limb paralysis, hemiparesis (weakness on one side of the body), akinesia (inability to move around), and abnormal movements, such as dikinesia (slight tremor of the hands) and orchoreo-athetosis (occurrence of involuntary movements). Hallucinations are also reported in rare cases. Without treatment, patients with stage II HAT slip into a coma and die (Brun *et al.*, 2010).

Nagana

Cattle and other livestock lack TLF, and are thus susceptible to *T. b. brucei*, which causes animal trypanosomiasis, also known as nagana (Baumgärtner, *et al.*, 2008). Nagana is a wasting disease of livestock and undermines the establishment of an efficient agricultural system in sub-Saharan Africa, resulting in estimated economic losses of ~\$4.5 billion dollars annually (FAO, Food and Agricultural Organization of the United Nations, 2007).

The primary clinical signs of nagana are intermittent fever, anemia, and weight loss. Cattle usually have a chronic infection, lasting a few weeks to several months depending upon the virulence. This infection may result in high mortality, especially if there is poor nutrition or other stress factors present. Some African cattle breeds are considerably more resistant to African trypanosomiasis. Susceptibility studies have shown these cattle have existed in the regions for over 5,000 years. The mechanism of trypano-tolerance has been demonstrated to have a genetic basis (Moulton and Sollod, 1976; Murray *et al.*, 1984).

Diagnosis

The CATT (Card Agglutination Test for Trypanosomiasis) is the most rapid serological test available for *T. b. gambiense*. Various patient samples such as serum, capillary blood from finger prick, and blood from impregnated filter papers are used with 87-98% sensitivity and 93-95% specificity (Noireau *et al.*, 1988; WHO, 1998; Jamonneau *et al.*, 2000; and Truc and Cuny, 2000). The CATT test relies on the presence of antibodies in patient sera to commonly occurring variants of *T. b. gambiense* surface proteins known as variant surface glycoproteins (VSGs). However, there is no serological test presently available for *T. b. rhodesiense*. The CATT is not a very effective diagnostic tool for *T. b. rhodesiense* as there are few commonly occurring VSGs that could be used. Hence, microscopic analysis of blood and/or lymph node aspirate is widely used for direct pathological confirmation of *T. b. rhodesiense* infection, as well as to

confirm *T. b. gambiense* infection. More advanced diagnostics such as immunofluorescence or enzyme-linked immunosorbent assays are generally used in non-endemic countries (WHO, 1998) (Lejon *et al.*, 1998). PCR-based diagnostics have the potential to provide a useful and sensitive method, though these tests need further validation and standardization (Chappuis *et al.*, 2005). Sensitive methods such as loop-mediated isothermal amplification (LAMP), which relies on amplification of a multicopy transposon-like sequence for detection of *T. brucei*, has promise as it can be used with specific primers for sub-species determination (Njiru *et al.*, 2008). However the biggest drawback is that these molecular diagnostic techniques are expensive, time-consuming, rely on more advanced infrastructure, and require highly trained personnel.

Treatment

Current treatment of HAT focuses on preventive strategies and chemotherapy. Preventive strategies are aimed at vector control, such as trapping, habitat destruction, and insecticide treatment. These methods can be cost effective, but are labor and time-intensive. The treatment of HAT depends on the stage of disease (I vs. II) and on the infective species (*gambiense* vs. *rhodesiense*) (WHO, 2010).

Stage I drugs

Pentamidine is used to treat stage I *T. b. gambiense* infection. It is administered intramuscularly or as a 2 h intravenous (IV) infusion every 24 hours

for 7 days. Pentamidine is well tolerated in patients and no resistance has been reported in the field. Some of the adverse reactions include hypoglycemia, injection site pain, diarrhea, and nausea. Pentamidine is transported into *T. brucei* by at least three known transporters. Pentamidine selectively binds to the minor groove of kinetoplast DNA, leaving the nuclear DNA unaffected. This promotes the cleavage of kinetoplast minicircle DNA, eventually leading to dyskinetoplastic cells (Barret *et al.*, 2007). Pentamidine is also a reversible inhibitor of S-adenosylmethionine decarboxylase, an enzyme in the polyamine biosynthetic pathway, but this is unlikely to be the primary mechanism of action (Barret *et al.*, 2007 and Wang, 1995).

Suramin is used to treat stage I *T. b. rhodesiense* infection. The dosing regimen is IV injections every 7th day for a month, with escalating dosage over the course of treatment (WHO 1986). Severe allergic reactions are often reported, and other side effects include albuminuria and hematuria (protein and blood in urine, respectively) and peripheral neuropathy. Suramin binds to *T. brucei* LDL receptors and thus, is rapidly endocytosed by the parasite. Suramin inhibits enzymes in the glycolytic and pentose phosphate pathways, resulting in rapid lysis of the parasite as the bloodstream form relies exclusively on glycolysis. Suramin also affects other enzymes such as thymidine kinase and dihydrofolate reductase (Wang, 1995).

Stage II drugs

Neither suramin nor pentamidine can be used for stage II *T. brucei* infection, as these drugs cannot penetrate the blood-brain barrier (Nok *et al.*, 2003). Until 1990, melarsoprol was the only drug available for the treatment of stage II HAT. It is given either as a series of 2-3 daily IV infusions for over a month or as single daily injections for 10 days (Chappuis, 2007). Like suramin, the mode of action of melarsoprol is not clear, but it is known to inhibit glycolytic enzymes such as pyruvate kinase, phosphofructokinase, and fructose-2,6-bisphosphatase (Wang *et al.*, 1995). Melarsoprol is also known to affect the redox balance of *T. brucei* by forming adducts with trypanothione (N1,N8-bisglutathionyl spermidine), a metabolite unique to trypanosomes (Wang *et al.*, 1995; Fairlamb *et al.*, 1985; Fairlamb *et al.*, 1989).

Melarsoprol, being an arsenic derivative, is very toxic to humans. Ten percent of patients administered this drug suffer from reactive arsenic-induced encephalopathy often followed by pulmonary edema and death within 48 h. Other common side effects include skin reactions such as pruritus (itching) and maculopapular eruptions (rash), peripheral motoric palsy (loss of movement), sensorial paraesthesia (loss of senses), neuropathies, and thrombophlebitis (vein inflammation). Highly insoluble in water, melarsoprol is dissolved in glycol, which also makes it very painful to administer (described as “fire in the veins”) and destroys veins after several administrations (Chappuis, 2007; Priotto *et al.*, 2006).

Difluoromethylornithine (DFMO), or eflornithine, is a more recent drug to treat stage II HAT, but it is only effective against *T. b. gambiense* infections. The drug is administered by IV infusion every 6 h for 14 days (Priotto *et al.*, 2006; Checchi *et al.*, 2007). Side effects of eflornithine treatment include bone marrow suppression, gastrointestinal symptoms, and convulsions, all of which subside upon completion of treatment (Chappuis *et al.*, 2004). Eflornithine is an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC), the initial enzyme in the polyamine synthetic pathway (Bacchi *et al.*, 1980). The major downsides of this drug are its cost, the complexity and duration of treatment, and its limited availability (Chappuis *et al.*, 2004).

Nifurtimox Eflornithine Combination Therapy (NECT), introduced in 2009, is the latest approach to treat stage II HAT. Nifurtimox is a registered drug for *T. cruzi* treatment and initial studies showed that the combination of eflornithine and nifurtimox was far superior to either eflornithine or melarsoprol and resulted in far less toxicity (Priotto *et al.*, 2007). The use of NECT also reduced the treatment regimen from 14 days to 7 days with a 94% cure rate. NECT has an additional benefit of easier administration, resulting in a reduction in staff and logistic resources. Combining two drugs also may reduce the possibility of the development of drug resistance. However, NECT is not effective for *T. b. rhodesiense* treatment (Kansiime *et al.*, 2009).

PARASITE LIFE CYCLE

T. brucei is transmitted as metacyclic trypomastigotes through the bite of the infected tsetse fly. After inoculation into the mammalian host, the parasites proliferate at the infection site, which can cause an inflamed nodule or ulcer (Tatibouet *et al.*, 1982), though the ulcers are rare with *T. b. gambiense* infection. Once in the bloodstream, *T. brucei* transforms into bloodstream form (BF) trypomastigotes that undergo rapid binary fission and populate the blood and lymph. Later during infection, BFs transform into non-dividing short stumpy forms, which are primed for return to the tsetse fly host. When a tsetse fly feeds on an infected mammal, the fly takes up the short stumpy forms with the blood meal (Fig. 1.2).

In the fly midgut, *T. brucei* transforms into procyclic form trypomastigotes (PFs), which also multiply by binary fission. The PFs then exit the midgut, traverse through the hemolymph, and migrate into the salivary glands, where they transform into epimastigotes. These epimastigotes undergo meiosis and sexual reproduction before undergoing a final transformation into metacyclic forms (Peacock *et al.*, 2011), which are ready for transmission to another mammalian host (Matthews, 2005; Fenn *et al.*, 2007; CDC, 2012).

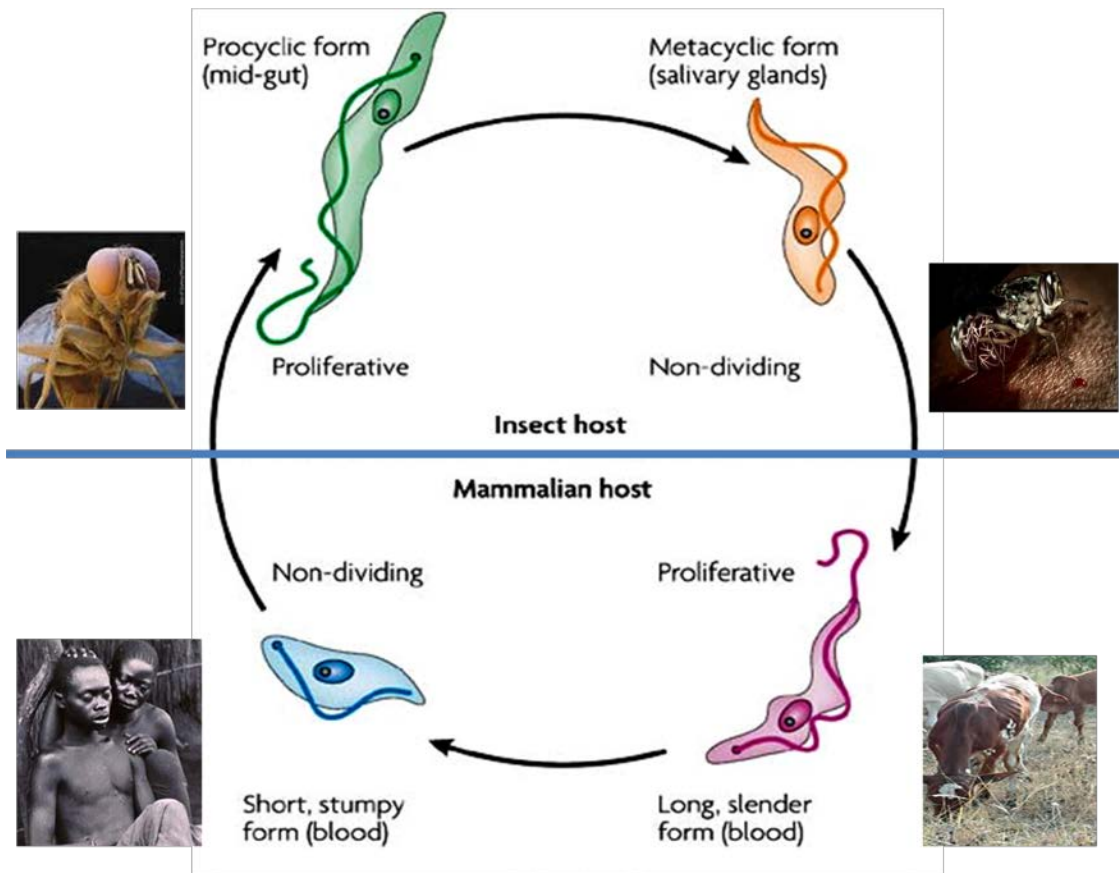


Figure 1.2: *T. brucei* life cycle. *T. brucei* cycles between its mammalian host and insect vector, the tsetse fly. Figure is modified from (Lee *et al.*, 2007).

PARASITE SURVIVAL STRATEGIES IN THE HOST

Parasite surface coats

In both the insect and mammalian stages, *T. brucei* expresses surface coat proteins that are important for its survival. The PF surface coat is composed of 10^6 copies of procyclin (Richardson *et al.*, 1988; Roditi *et al.*, 1989), a glycosylphosphatidylinositol (GPI)-anchored protein containing characteristic amino acid repeats at the C terminus: either Glu-Pro repeats (EP procyclin) or

Glu-Pro-Glu-Glu-Thr repeats (GPEET procyclin). The procyclin GPI anchor contains one C16:0 (palmitate) and one C18:0 (stearate) fatty acid as the lipid moiety (Butikofer *et al.*, 1997). Procyclins are predicted to play a role in protecting these parasites against proteolytic degradation in the tsetse fly midgut (Acosta-Serrano *et al.*, 2001).

In the mammalian bloodstream, *T. brucei* is fully exposed to the immune system, but has multiple strategies to evade the host's immune responses. The BF surface coat protein VSG plays a key role in the parasite's ability to evade the mammalian immune system. VSGs are also GPI-anchored to the plasma membrane, and the lipid moiety consists of two C14:0 fatty acids (myristate). VSGs are highly immunogenic and form a dense coat around the parasite with 10^7 copies per cell. The VSG coat protects the invariant surface antigens from immune recognition and stimulates a strong antibody response. Periodic shedding of VSG coat provides protection from complement-mediated lysis (Tachado and Schofield, 1994; Taylor and Rudenko, 2006).

The *T. brucei* genome possesses >1500 different VSG genes and ~20 different VSG expression sites (Barry *et al.*, 2005; Berriman *et al.*, 2005; Marcello *et al.*, 2007). Only one VSG is expressed at a time, a process tightly regulated by an allelic exclusion mechanism. At any one time, parasites expressing the dominant VSG coat are lysed and killed by the host immune system, and a subset of the parasite's population switches its coat to a different VSG before being killed by the immune system (Turner and Barry, 1989). This periodic VSG

switching forces the immune system to mount successive waves of specific antibodies against the distinct VSGs (Borst *et al.*, 2002). Hence the immune system can never completely destroy the parasite and the parasite population persists in the host, ultimately causing severe symptoms and death of the host if untreated.

Evasion of complement mediated lysis

A ~10-fold higher endocytosis rate is observed in BFs compared to PFs (Natesan *et al.*, 2007; Field and Carrington, 2009). This upregulation of endocytosis clears antibodies bound to the parasite surface, thereby evading the antibody-mediated complement cascade and resulting death by cell lysis. Upregulation of both fluid phase endocytosis and receptor-mediated endocytosis is associated with the clearance of antibodies and complement from the surface (Barry, 1979; Ferrante and Allison, 1983; Pal *et al.*, 2003; Engstler *et al.*, 2007). Upregulation of fluid phase endocytosis is responsible for the high general membrane turnover rate observed in BFs (i.e. complete VSG turnover occurs in ~12 minutes) (Engstler *et al.*, 2004), whereas upregulation of receptor-mediated endocytosis is responsible for clearing surface-bound antibodies and complement from the parasite surface via VSG recycling (Kabiri and Steverding, 2000; Chung *et al.*, 2004; Field and Carrington, 2009). VSG bound to antibodies and complement are internalized via the flagellar pocket into a VSG recycling endosome, which contains proteases that selectively degrade and inactivate the antibodies and complement. The VSG is then recycled back to the surface.

Modulation of host immunity

Trypanosomes are sensitive to reactive oxygen and nitrogen species, a common immune killing mechanism. In the bloodstream, *T. brucei* triggers a cascade of immune reactions modulated for the benefit of the parasite (Shi *et al.*, 2004; Pan *et al.*, 2006). *T. brucei* activates polyclonal B cell expansion, which induces the production of IgM antibodies. The parasites are opsonized by IgM (and IgG) antibodies, which activates macrophage recruitment and leads to the production of inflammatory cytokines. These inflammatory cytokines then down-regulate the production of reactive oxygen and nitrogen species. At the same time, *T. brucei* suppresses the development and function of T lymphocytes, which results in an overall immune suppression (Barrett *et al.*, 2003). *T. brucei* also secretes immunomodulatory molecules (Trypanokines) that mimic the activities of host cytokines (Vaidya *et al.*, 1997) and are capable of triggering immune responses that are not required for elimination of the parasite itself (Sharafeldin, 1999) but favors survival and persistence of *T. brucei* and establishment of later stages of infection (Hamadien *et al.*, 1999). These immunomodulatory tactics provide the parasite additional mechanisms to evade the host immune system (Lucas *et al.*, 1994).

HOST MICROENVIRONMENTS

In the different stages of its life cycle, *T. brucei* encounters a number of distinct microenvironments. In the insect, the parasite encounters the tsetse

midgut, ectoperitrophic space, and salivary glands; while in the mammal, the parasite encounters the blood, lymph, and cerebrospinal fluid (Fig. 1.3). These environmental niches differ in their nutrient composition, including lipids. For example, when *T. brucei* leaves the bloodstream and crosses the blood-brain barrier, it experiences a dramatic ~300-fold drop in environmental lipids (Roheim *et al.*, 1979).

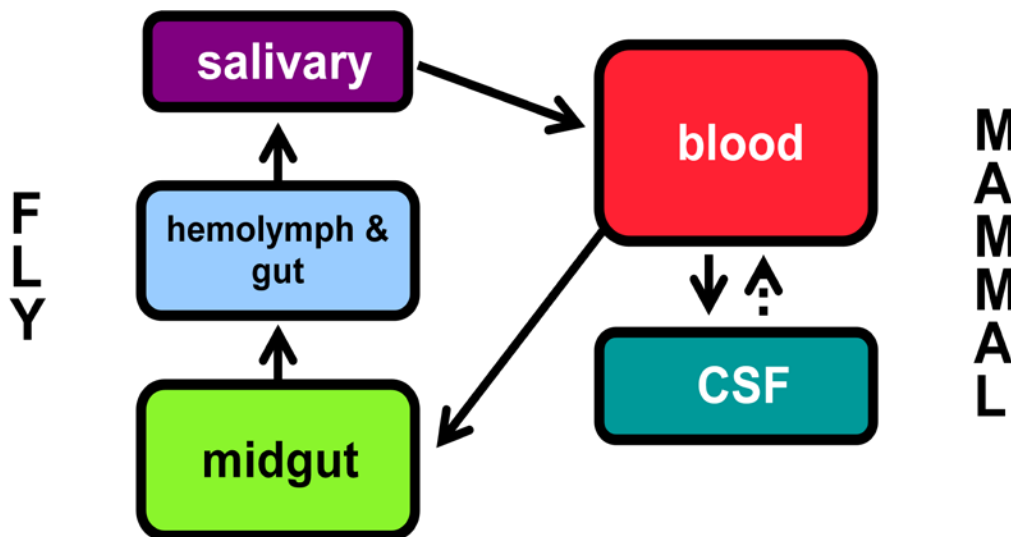


Figure 1.3: Host microenvironments. *T. brucei* encounters different microenvironmental niches in its tsetse fly and mammalian hosts, which differ in their nutrient composition including fatty acids.

Fatty acids (FAs) are the simplest lipids and they play a major role in *T. brucei*. Not only are FAs structural components of membranes, but they are also cell signaling molecules and play a vital role in protein localization via membrane anchors. In particular, FAs are key constituents of GPI-anchors that localize

surface proteins like VSG and Procyclin. The high density of these GPI-anchored coat proteins creates a high demand for FAs in *T. brucei*.

T. brucei can effectively take FAs from the host or synthesize their own to satisfy its fatty acid requirements. If FAs in the host microenvironment become limiting, then *T. brucei* must rely on FA synthesis. Synthesis of FAs requires more energy than uptake, thus *T. brucei* must have the ability to efficiently regulate *de novo* FA synthesis to meet its changing nutrient demands (Fig. 1.4). Earlier studies in our lab demonstrated that a growth defect was observed in PFs when FA synthesis was knocked down in cells grown in low lipid conditions (Vigueira *et al.*, 2011). Knock down of FA synthesis also reduced virulence in a mouse model of infection (Vigueira *et al.*, 2011). A high demand for FAs is also created by the need to maintain a high membrane turnover required to avoid antibody-mediated complement lysis (McKnight, 2012).

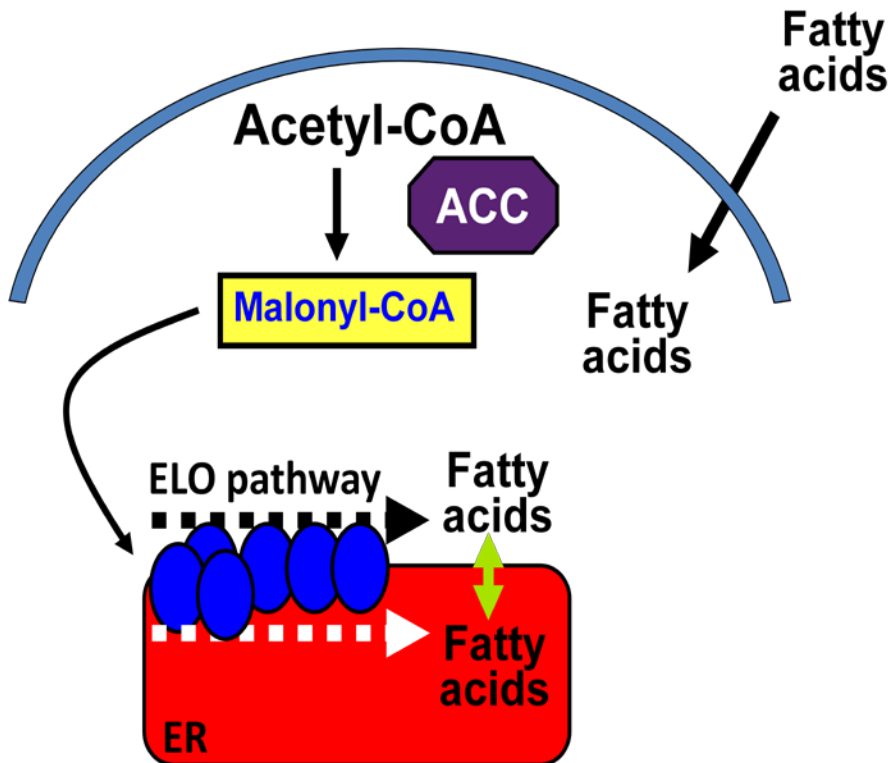


Figure 1.4: Fatty acid synthesis vs. uptake. *T. brucei* can effectively take up FAs when environmental lipids are available. However, under conditions of limited external lipids, *T. brucei* synthesizes FAs by converting acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). The malonyl-CoA is then used by the ELO pathway for FA synthesis.

FATTY ACID UPTAKE

One mode of lipid uptake in various systems is the endocytosis of lipoproteins. Lipoproteins have a polar surface layer made up of phospholipids, apolipoproteins and cholesterol; and a non-polar inner core comprised of triglycerides, cholesterol, and cholesterol esters (Wasan *et al.*, 2008). After internalization, the lipoproteins are transported to lysosomes where they are

degraded and the lipids are liberated. In addition, lipoproteins are also acquired directly from donor lipoprotein particles without being transported to lysosomes (Engelmann and Weidmann, 2010).

A second major lipid uptake mechanism involves the direct uptake of phospholipids and FAs (Glatz *et al.*, 2010). Due to their low aqueous solubility, these lipids often associate with carrier proteins (typically serum albumin) in the bloodstream. The internalization of lipids by cells begins with their dissociation from carrier proteins and partitioning into the outer leaflet of the plasma membrane bilayer. This is followed by flip-flop movement of the phospholipids and FAs, which changes the orientation of the polar head group from the outer to the inner lipid-water interface. This process enables FAs to partition into the cell, where they become bound or activated, and ultimately incorporated into more complex lipids. This process follows biphasic kinetics: partitioning into the lipid bilayer is much faster, and internalization is much slower. The process of lipid flip-flop, being the slowest step in FA uptake, is considered the rate-limiting step (Mellors *et al.*, 1989; Kamp *et al.*, 2007a; Kamp *et al.*, 2007b).

The transmembrane protein, CD36, and plasma membrane FA binding protein (FABP) both facilitate FA diffusion by binding and concentrating FAs on the membrane surface (Ehehalt *et al.*, 2006). The bound FAs are then more easily partitioned into the membrane, resulting in a greatly enhanced FA uptake rate. FA uptake also takes place through lipid rafts associated with caveolae (Glatz *et al.*, 2010). Caveolin binds FAs with high affinity, but the major role of

caveolin in FA uptake is likely by providing a docking site for CD36. Supporting this idea, cholesterol depletion leads to the disassembly of lipid rafts, the dispersion of CD36 throughout the plasma membrane, and a resulting decrease in FA uptake (Ehehalt *et al.*, 2006; Glatz *et al.*, 2010). In addition to passive diffusion, FA uptake is also mediated by a family of FA transport proteins (FATPs), which are integral membrane proteins responsible for active transport of FAs into cells (Glatz *et al.*, 2010).

FA uptake can also occur through the action of proteins not associated with the plasma membrane. FAs must be activated before they can be metabolized for energy or incorporated into more complex lipid species. FA activation occurs by the esterification of a FA to Coenzyme A (CoA), mediated by a family of enzymes called acyl-CoA synthetases (ACS) (Webster, 1963). Failure to activate FAs can result in free outward passive diffusion and no net uptake. Activation of the FA to the CoA form makes the FA membrane impermeable, and thus, retention of FAs internalized by facilitated or passive diffusion is enhanced by ACSs (Milger *et al.*, 2006).

Fatty acid uptake in *T. brucei*

Various mechanisms of FA uptake have been described in *T. brucei*. Endocytosis of high density and low density lipoproteins by BFs is well described (Coppens *et al.*, 1995; Green *et al.*, 2003). The lipoprotein scavenger receptor enhances this process (Green *et al.*, 2003; Thomson *et al.*, 2009). A similar endocytic mechanism used to take up lipophorin, a family of high density

lipoproteins in the fly, has been demonstrated in the related trypanosome, *T. rangeli* (Folly *et al.*, 2003). Specialized uptake mechanisms for the acquisition of phospholipids occurs along with lipoprotein uptake in *T. brucei*. For example, the coordinated activity of three proteins: phospholipase A1, acyl-CoA ligase, and lysophosphatidylcholine: acyl-CoA acyltransferase has been confirmed in the uptake of lysophosphatidylcholine (Bowes *et al.*, 1993).

Little is known about the mechanisms of FA uptake in *T. brucei*. Proteins involved in the uptake of FAs have not yet been identified or characterized in these parasites, though early studies demonstrate that FA uptake occurs with biphasic kinetics (Voorheis, 1980). This kinetic similarity between mammalian and *T. brucei* FA uptake suggests the presence of both protein-mediated active transport and passive diffusion in trypanosomes. The genome of *T. brucei* encodes 5 ACSs, four of which have been characterized (Jiang *et al.*, 2000; Jiang *et al.*, 2001; Jiang *et al.*, 2004). The effect of TbACS knock-down by a PAN ACS construct was measured by monitoring uptake of a fluorescently tagged C12:0 FA (BODIPY-C12:0). The uptake of BODIPY-C12:0 was reduced by 34% in 2 days, 47% in 4 days, and 58% in 6 days upon RNAi induction. However panRNAi of the TbACSs caused only a small reduction in growth (Vigueira, 2011).

FA synthesis in *T. brucei*

For many years, only PF *T. brucei* were thought to possess the ability to synthesize FAs while BF lacked this capacity (Dixon *et al.*, 1971). However,

studies showed that both BF and PF can synthesize FAs *de novo* from a butyryl-CoA primer (Morita *et al.*, 2000). Subsequently, the genome project of *T. brucei* revealed the absence of a canonical eukaryotic type I FA synthase (Berriman *et al.*, 2005). Instead, *T. brucei* possesses a microsomal elongase (ELO) FA synthesis pathway and mitochondrial type II FA synthesis pathway. *T. brucei* relies on the microsomal ELO pathway for the bulk of their FA synthesis (Lee *et al.*, 2006) in contrast to other eukaryotic organisms, where the ELO pathway is used for elongation of pre-existing long chain FAs. *T. brucei* possesses four ELO genes (ELO1-4) encoding the ketoacyl-CoA synthases of the ELO pathway. Each of these four ketoacyl-CoA synthases have distinct, but overlapping chain-length specificities: ELO1 (C4:0–C10:0), ELO2 (C10:0–C14:0), and ELO3 (C14–C18), and ELO4 acts on long chain unsaturated FAs (Lee *et al.*, 2006). The ELO pathway also comprises two ketoacyl-CoA reductases (KCR), which reduce ketoacyl-CoA to hydroxylacyl-CoA; a dehydratase that converts hydroxyacyl-CoA to enoyl-CoA; and an enoyl-CoA reductase that converts enoyl-CoA to an acyl-CoA two carbons longer than the primer chain. Individual ELOs are not essential *in vivo*, likely due to their overlapping substrate specificities. However, RNAi of the sole enoyl-CoA reductase that acts downstream of all the ELOs suggests that the ELO pathway as a whole is essential (Lee *et al.*, 2006) (Fig. 1.5).

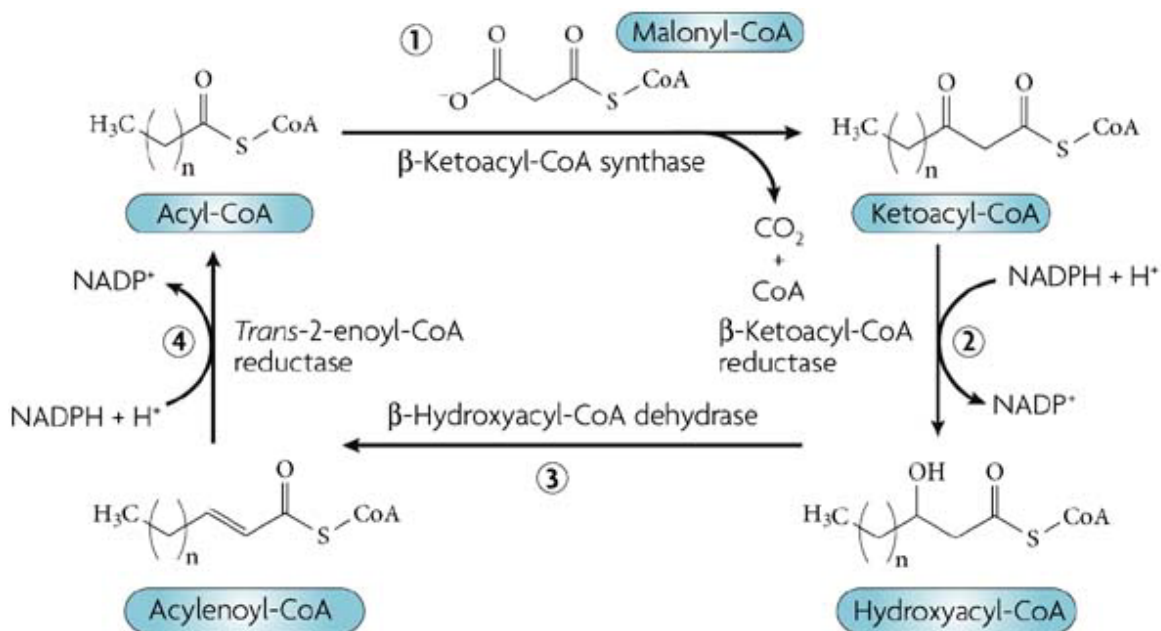


Figure 1.5: Enzymatic reactions of FA synthesis pathway in *T. brucei*. The *T. brucei* ELO FA synthesis pathway consists of four reactions: the 2-carbon donor malonyl-CoA is condensed with a butyryl-CoA primer by ketoacyl-CoA synthase (1), reduced by ketoacyl-CoA reductase (2), dehydrated by dehydratase (3), and finally reduced by enoyl-CoA reductase (4) to yield a FA product that 2 carbons longer. The malonyl-CoA is synthesized from acetyl-CoA by acetyl-CoA carboxylase (ACC). Figure is modified from (Lee *et al.*, 2007).

The mitochondrial type II FA synthesis pathway is composed of one acyl carrier protein (ACP), one ketoacyl-ACP synthase, three ketoacyl-ACP reductases, a dehydratase, and two enoyl-ACP reductases (Stephens *et al.*, 2007; Autio *et al.*, 2008). The major products synthesized by the mitochondrial type II pathway are C16:0 and C8:0, the latter of which is the precursor for lipoic acid, a key prosthetic group in several mitochondrial respiratory enzymes. Mitochondrial FA synthesis is known to account for only ~10% of overall FA

synthesis in *T. brucei*, but this pathway is essential in both PFs and BFs to supply FAs required for lipoic acid synthesis and to locally maintain mitochondrial membrane composition, respiratory complex functions, and mitochondrial morphology (Stephens *et al.*, 2007; Guler *et al.*, 2008).

In addition to being found in other trypanosomes such as *T. cruzi*, ELO pathways are present in other parasites including *Leishmania major*, *Plasmodium falciparum*, *Toxoplasma gondii*, and *Entamoeba histolytica*, though their exact functions may vary (Lee *et al.*, 2007). The modular structure of the ELO pathway in trypanosomatids enables differential control of the FA products. This may be advantageous to allow parasites to adapt to different host environments. For example, downregulation of ELO3 might be responsible for the observed chain-length preference in BFs to synthesize myristate (C14:0), which is the sole FA component of the VSG GPI-anchor (Lee *et al.*, 2006; Lee *et al.*, 2007). The presence of environmental lipids can also lead to the overall up- and downregulation of the ELO pathway to meet the FA demands of the parasite. For example, in low lipid conditions the balance shifts towards FA synthesis and increased ELO pathway activity. In extreme cases, BF will even elongate myristate, normally the final product of FA synthesis in BFs (Doering *et al.*, 1993; Lee *et al.*, 2006). The exact mechanisms regulating the ELO and mitochondrial FA synthesis pathways and the roles they play in *T. brucei* pathogenesis are largely unknown.

ACETYL-COA CARBOXYLASE

Acetyl-CoA carboxylase (ACC) is a key enzyme in the FA synthesis pathway and catalyzes the first committed step: the conversion of acetyl-CoA to malonyl-CoA, the 2-carbon donor for FA synthesis (Barber *et al.*, 2005). ACC uses a biotin prosthetic group to transfer CO₂ from bicarbonate to acetyl-CoA in two half reactions: biotin carboxylation and carboxyl transfer. In bacteria, archaea, and chloroplasts, ACC is a multi-enzyme complex composed of a biotin carboxylase (BC) enzyme; a biotin carboxyl carrier protein (BCCP) that carries the biotin prosthetic group attached to a conserved lysine; and a carboxyltransferase enzyme (CT) composed of one or two subunits (Fig. 1.6). In contrast, many eukaryotic ACCs are large multi-domain enzymes composed of BC and CT domains separated by a BCCP domain (Fig. 1.6).

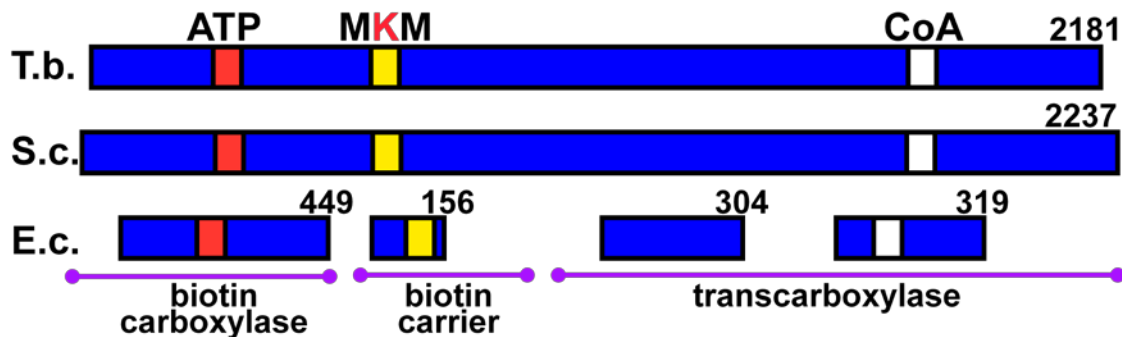


Figure 1.6: Comparison of ACC protein structure. T.b., *T. brucei* ACC; S.c., *Saccharomyces cerevisiae* ACC; E.C., *Escherichia coli* ACC.

Known ACC functions

The function of ACC depends on the role of its product, malonyl-CoA. The majority of metazoans express two different ACC isoforms that differ in their

functions (Barber *et al.*, 2005): ACC1 (ACCA) initiates FA synthesis in lipogenic tissues, whereas ACC2 (ACC β) is expressed in vertebrate oxidative tissues such as skeletal muscle and heart. ACC2 (ACC β) is associated with the mitochondria and plays a role in regulating FA oxidation (Abu-Elheiga *et al.*, 1997; Abu-Elheiga *et al.*, 2000). Malonyl-CoA synthesized by ACC2 inhibits carnitine palmitoyltransferase I, part of the machinery that imports FAs into the mitochondria for oxidation. In tissues such as the liver, both ACC isoforms are expressed and function coordinately (Abu-Elheiga *et al.*, 1997; Abu-Elheiga *et al.*, 2001; Mao *et al.*, 2006). In the hypothalamus, ACC also functions as a component of a malonyl-CoA dependent fuel sensing pathway that regulates whole body metabolism (Hu *et al.*, 2003; Hu *et al.*, 2005; Wolfgang and Lane, 2006).

In most unicellular organisms, malonyl-CoA formation is the first committed step in FA synthesis. *S. cerevisiae* ACC is the best characterized among unicellular eukaryotes. This organism harbors two ACCs: a cytosolic ACC that functions in FA synthesis and elongation, and a mitochondrial ACC that functions in lipoic acid synthesis (Hoja *et al.*, 2004; Hiltunen *et al.*, 2005). Unlike mammalian ACC2, the *S. cerevisiae* mitochondrial ACC does not play a role in regulating FA oxidation, perhaps because FA oxidation occurs in specialized organelles known as peroxisomes rather than mitochondria (Hiltunen *et al.*, 2005). Very little is known about ACC in protozoan parasites. The Apicomplexans *Toxoplasma* and *Plasmodium* express two multi-domain ACCs.

The ACC in the apicoplast (a non-photosynthetic plastid organelle) plays an essential role in lipoic acid synthesis (Jelenska *et al.*, 2001), however the function of Apicomplexan cytosolic ACC is not clearly understood (Jelenska *et al.*, 2001; Gornicki, 2003; Mazumdar *et al.*, 2006). Another Apicomplexan, *Cryptosporidium*, expresses only single multidomain ACC, which is located in cytoplasm (Zhu, 2004; Zhu *et al.*, 2000).

***T. brucei* ACC**

According to the TriTryp database, the *T. brucei* genome encodes a single predicted ACC isoform (Tb927.8.7100) (Aslett *et al.*, 2009). Southern blot analysis confirmed the presence of a single copy gene (Vigueira *et al.*, 2011). The *T. brucei* ACC (TbACC) gene is ~6.5 Kb in length and encodes a protein of 2,181 amino acids with a calculated molecular weight 243.9 kD. TbACC shares the multidomain structure of other eukaryotic ACCs, and multiple sequence alignments showed that TbACC has a moderate 30% identity to yeast and human ACCs, and a much higher ~60% identity to other trypanosomatid ACCs (Vigueira *et al.*, 2011). TbACC is expressed in both BFs and PFs. The expression of TbACC in PFs is 6-fold higher than in BFs when normalized to tubulin, which is consistent with a 5-fold higher rate of FA synthesis in PFs compared to BFs (Morita *et al.*, 2000; Vigueira *et al.*, 2011).

TbACC was predicted to be cytosolic and sub-cellular localization experiments confirmed this prediction (Vigueira *et al.*, 2011). However, immunofluorescence microscopy showed that TbACC is localized to a multitude

of small distinct puncta rather than in a diffuse cytoplasmic distribution (Vigueira *et al.*, 2011). RNA silencing of TbACC resulted in partial inhibition (50-70%) of [³H]laurate and [³H]myristate elongation in PFs. In BFs, TbACC RNAi caused only a 15% inhibition of [³H]laurate elongation, but completely abolished the minor elongation of [³H]myristate. When grown in normal lipid conditions, TbACC RNAi cells exhibited no growth defect in either PFs or BFs, which could be attributed to the ability of the parasite to acquire FAs from its environment (i.e. media). However, when the environmental conditions were changed to low lipid conditions, TbACC RNAi in PFs reduced growth by 64% (Vigueira *et al.*, 2011). Though TbACC RNAi had no effect on growth of BFs even in low lipid conditions, it reduced virulence in mice, nearly doubling the mean time to death when compared to the uninduced cell line where TbACC was expressed normally.

Regulation of ACC

In mammals, ACC is highly expressed in certain tissues, such as white and brown fat, liver, and lactating mammary glands, but ACC expression is inhibited during starvation (Wakil *et al.*, 2009). Three distinct promoters are known to play a role in controlling the expression of ACC at the transcriptional level in response to glucose, hormones, and leptins (Hillgartner and Charron, 1997; Mao *et al.*, 2003; Kim *et al.*, 2010). Transcription factors such as sterol-regulatory-element-binding protein 1c (SREBP1c), X-receptor, retinoid X receptor, and peroxisome-proliferator-activated receptors (PPARs) play an

important role in controlling ACC1 expression (Shimano *et al.*, 1999; Wolfram *et al.*, 2004; Barber *et al.*, 2005).

Allosteric effectors, such as citrate and other carboxylic acids, are known to activate mammalian ACC (Vagelos *et al.*, 1963; Lane *et al.*, 1974). Signals from amino acid metabolism such as glutamate also activate ACC1 (Winz *et al.*, 2000). Malonyl-CoA, free CoA and FA-CoA esters were also demonstrated to inhibit ACC1 (Ogiwara *et al.*, 1978; Moule *et al.*, 1992).

Metabolic labeling experiments on liver and fat tissues with [³²P]phosphate demonstrated the presence of at least four hormone-responsive phosphorylation sites in ACC. Treatment with glucagon leads to the rapid phosphorylation of ACC, which is an inactivating post-translational modification (Brownsey *et al.*, 1992; Hardie *et al.*, 1992). Phosphorylation of ACC is mediated by two major kinases: AMP-dependent protein kinase (AMPK), and protein kinase A (PKA) (Hardie *et al.*, 1992).

Many metabolic enzymes are organized in complexes known as 'metabolons', where high intracellular protein concentrations favor protein-protein interactions (Velot *et al.*, 1997). Mammalian and avian ACC activity is regulated by polymerization: ACC exists as dimeric form but can assemble into a higher-order polymeric form (Kleinschmidt *et al.*, 1969; Mackall *et al.*, 1978; Thampy *et al.*, 1985; Barber *et al.*, 2005). Allosteric activators such as citrate induces *in vitro* polymerization of ACC in chicken liver extracts, where the inactive protomeric form of the enzyme converts to an active filamentous form composed of 10-20

protomers (Beaty *et al.*, 1983). In addition, protein inducers like MIG12 can induce ACC polymerization in mice by lowering the threshold citrate concentration (Kim *et al.*, 2010). Negative regulators such as malonyl-CoA, ATP, and Mg^{2+} causes depolymerization and inactivation of fully polymerized ACC (Beaty *et al.*, 1983).

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CHAPTER TWO

REGULATION OF *T. brucei* ACC IN PRESENCE OF ENVIRONMENTAL LIPIDS

INTRODUCTION

African trypanosomiasis is caused by the parasite *Trypanosoma brucei*, which is transmitted by the blood sucking tsetse fly. In mammals, the bloodstream form (BF) of the parasite exists extracellularly in the bloodstream and the cerebrospinal fluid. When the tsetse fly feeds on an infected mammal, *T. brucei* differentiate into procyclic forms (PF) in the fly midgut. These PFs then invade the hemolymph, cross the midgut epithelium and enter into the salivary glands. In the salivary glands, the PFs undergo meiosis and further development to form an infectious metacyclic form, which is transmissible to a new mammalian host when the fly takes another blood meal.

T. brucei surface coat proteins play an important role in the survival of the parasite in its hosts. The *T. brucei* PF surface coat is composed of $\sim 3 \times 10^6$ copies of one of the procyclins, a small family of glycoproteins (Schell and Overath, 1990; Matthews and Gull, 1994). Procyclins are predicted to play a role in protecting these parasites against proteolytic degradation in the tsetse fly midgut (Acosta-Serrano *et al.*, 2001). The *T. brucei* BF surface coat is composed of 1×10^7 copies of a single Variant Surface Glycoprotein (VSG) (Tachado and Schofield, 1994; Taylor and Rudenko, 2006). *T. brucei* can periodically switch its

VSG coat to an entirely new antigenic variant thereby allowing the parasite to avoid destruction by the immune system, a process known as antigenic variation (Turner and Barry, 1989). Both the procyclin and VSG proteins are GPI-anchored to the parasite plasma membrane, though their GPI-anchors differ in their fatty acid (FA) moieties. The procyclin GPI-anchor contains two FAs, a palmitate (C16:0) esterified to inositol and a stearate (C18:0) esterified to the sn-1 position of the monoacylglycerol moiety (Field *et al.*, 1991; Butikofer *et al.*, 1997). The VSG GPI-anchor also contains two FAs, both of which are myristate (C14:0) (Ferguson and Cross, 1984). Consequently, the need to maintain their lipid anchored surface coats creates a high demand for FAs. Interestingly, myristate is not an abundant FA in blood. (Edelstein, 1986).

During its life cycle, a host presents *T. brucei* with different microenvironments: the blood meal, hemolymph, gut and salivary glands in tsetse flies; and the bloodstream and cerebrospinal fluid in mammals. In all of these microenvironments, the parasite encounters differences in nutrient availability, including FAs. For example, the parasite experiences a dramatic ~400-fold drop in lipid availability when it crosses the blood-brain barrier and enters the cerebrospinal fluid (Roheim *et al.*, 1979). To satisfy its FA needs, *T. brucei* relies on two mechanisms: *de novo* synthesis of FAs and uptake of environmental FAs from the host. *T. brucei* readily takes fatty acids from its host to supply itself (Mellors and Samad, 1989; Coppens *et al.*, 1995). However, under conditions of limiting exogenous FAs, *T. brucei* is unable to rely on uptake

alone and must synthesize FAs *de novo*. FA synthesis is functional in both PF and BF *T. brucei* (Lee *et al.*, 2006; Stephens *et al.*, 2007).

A canonical eukaryotic cytosolic type 1 pathway does not exist in trypanosomatids (Aslett *et al.*, 2010). Instead, there are two known FA synthesis pathways in *T. brucei*: an elongase pathway localized to the endoplasmic reticulum that accounts for ~90% of total FA synthesis (Lee *et al.*, 2006) and a mitochondrial pathway that is responsible for ~10% of total FA synthesis (Stephens *et al.*, 2007; Guler *et al.*, 2008). Both FA synthesis pathways depend on a key substrate malonyl-CoA, the 2-carbon chain donor, which is synthesized from acetyl-CoA by Acetyl-CoA carboxylase (ACC) in what constitutes the first committed step in FA synthesis (Barber *et al.*, 2005).

T. brucei ACC (TbACC) is 58-66% identical to the ACCs in other trypanosomatids such as *Leishmania* and *Trypanosoma cruzi* (Vigueira *et al.*, 2011). However, TbACC is only 31-33% identical to human, rat, and *Saccharomyces cerevisiae* ACCs (Vigueira *et al.*, 2011). ACC is a member of the biotin carboxylase family of enzymes, which use a biotin prosthetic group to transfer a carboxyl group to an acceptor acetyl-CoA (Barber *et al.*, 2005). The domain identity between the human ACC and TbACC is ~ 48% for the biotin carboxylase domain, ~30% for the biotin carboxyl carrier protein domain, and ~34% for the carboxyl transferase domain (Vigueira *et al.*, 2011). Earlier studies demonstrated that RNAi of TbACC in PFs and BFs caused a reduction in FA elongation (Vigueira *et al.*, 2011). TbACC RNAi also caused a growth defect in

PFs when environmental lipids were low, and reduced the virulence of BFs in mouse infection studies (Vigueira *et al.*, 2011).

Because ACC catalyzes the first committed step in FA synthesis, it serves as a control point for regulating FA synthesis and is known to be highly regulated by various control mechanisms in other organisms (Brownsey *et al.*, 2006). In many metazoans, ACC is a component of a fuel-sensing pathway that responds to the metabolic state of cell (Hu *et al.*, 2003; Hu *et al.*, 2005; Wolfgang and Lane, 2006). In all systems studied to date, ACC is regulated by phosphorylation. AMP-activated protein kinase (AMPK) is the best characterized kinase that phosphorylates ACC, and its function is conserved from yeast to mammals where it serves as an energy and stress sensor (Winder and Thomson, 2007). In yeast and mammals, phosphorylation of ACC results in the inhibition of ACC activity, creating a way to conserve energy during stress (Winder *et al.*, 1997; Carlson and Winder 1999). Mammalian ACC is phosphorylated on least 6-8 sites, however only 2-3 sites have actually been demonstrated to affect ACC activity (Tong, 2005). In mammals, the canonical sites for AMPK phosphorylation are Ser79, Ser1200, and Ser1215, whereas yeast ACC is primarily controlled by AMPK phosphorylation at Ser1500 (Barber *et al.*, 2005).

ACC likely plays a central role in FA metabolism in *T. brucei*, however little is known about ACC in parasitic protozoans and almost nothing is known about trypanosomatid ACCs and their regulation. In this chapter, I investigated the hypothesis that *T. brucei* ACC is regulated in response to environmental lipids. I

found that ACC activity is increased in low lipid environments and decreased in high lipid environments. I then delineated the mechanisms by which ACC expression and activity is regulated in response to altered lipid environments and found that phosphorylation of TbACC has a negative effect on TbACC activity and is one of the major post-translational mechanisms regulating TbACC in response to environmental lipids. Finally, I found that environmental lipid regulation of TbACC occurs only in PFs and is not observed in BFs.

RESULTS

Effect of environmental lipids on TbACC mRNA levels

To examine the regulation of TbACC at the transcriptional level, we used qRT-PCR (quantitative reverse transcriptase polymerase chain reaction). Total mRNA was isolated from lysates prepared from cells grown in low, normal, and high lipid conditions. qRT-PCR analysis revealed that in both BFs and PFs, there was no significant difference in TbACC mRNA levels in cells grown in low, normal, and high lipid conditions (Fig. 2.1 A). There is no significant difference in TbACC mRNA level between PF and BF when normalized to actin (Fig. 2.1 B).

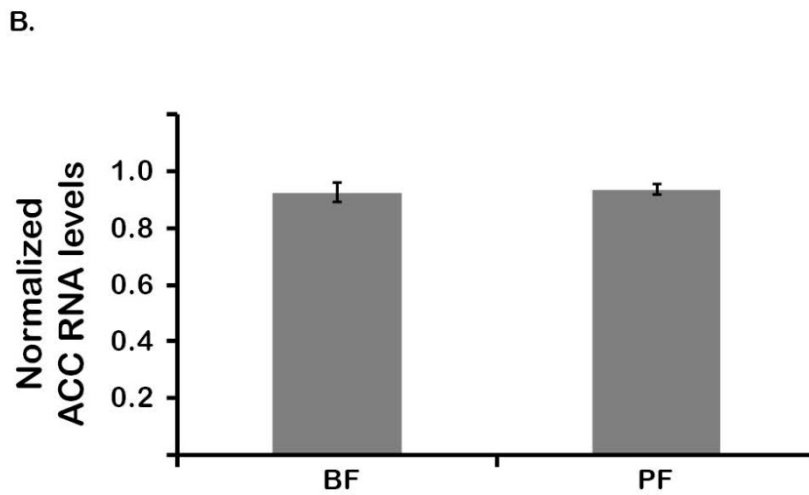
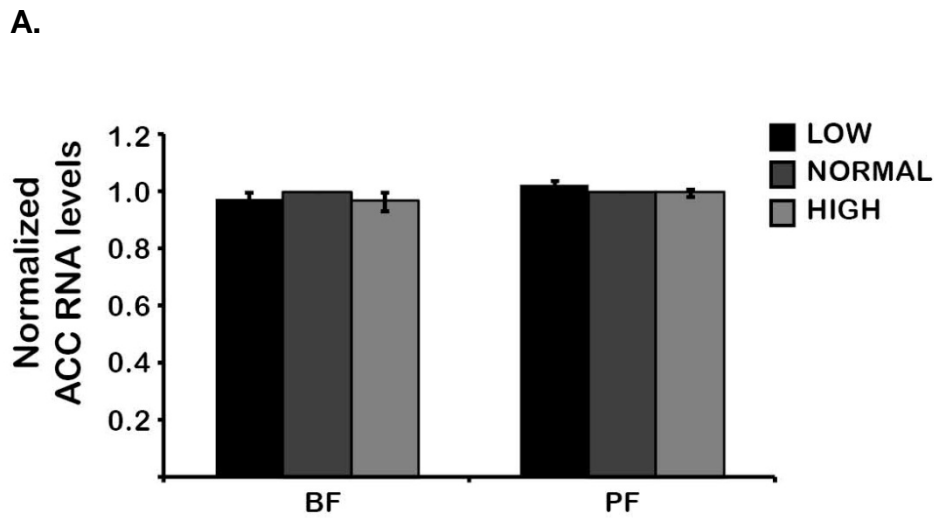


Figure 2.1: Environmental lipids do not affect ACC mRNA levels in PF and BF. (A.) BF and PF *T. brucei* cells were grown in low, normal, and high lipid conditions to mid-logarithmic stage. ACC transcript levels were assessed by qRT-PCR and normalized to β -actin as a loading control. Data shows the mean \pm SEM of three independent experiments. **(B.)** BF and PF ACC RNA levels normalized to actin as a control.

Effect of environmental lipids on TbACC protein levels

To investigate regulation of TbACC at the post-translational level, BF and PF cells were grown in low, normal, and high lipid media and then TbACC protein levels in total cell lysates were analyzed by SDS-PAGE and western blotting using streptavidin-HRP (SA-HRP), which binds to the biotin prosthetic group of ACC (Nikolau *et al.*, 1985; Haneji and Koide, 1989). Other biotinylated proteins can also be detected using SA-HRP, however the genome of *T. brucei* shows the presence of only one other biotinylated protein, the 71 kD α subunit of 3-methylcrotonyl-CoA carboxylase (Tb927.8.6970), an enzyme involved in amino acid degradation. TbACC is 243 kD, making it distinguishable from the other biotinylated enzyme in *T. brucei*. In BFs, TbACC protein levels showed no significant difference in cells grown in low, normal, or high lipid media (Fig. 2.2.A and C). However, in PFs growth in low lipid media resulted in a significant 1.6-fold increase in TbACC protein levels as compared to high lipid media (Fig. 2.2.B and C).

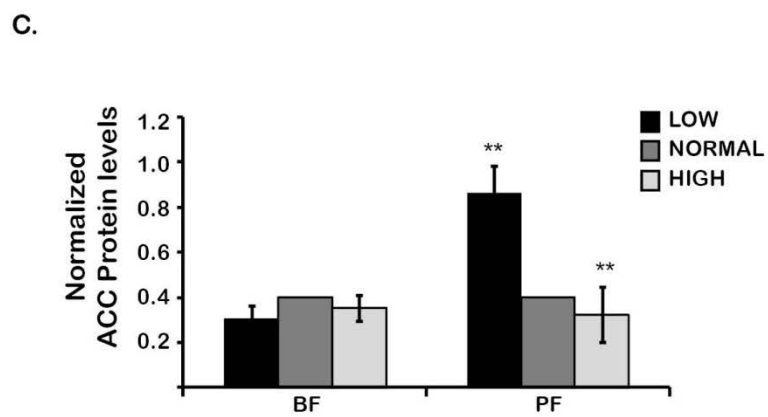
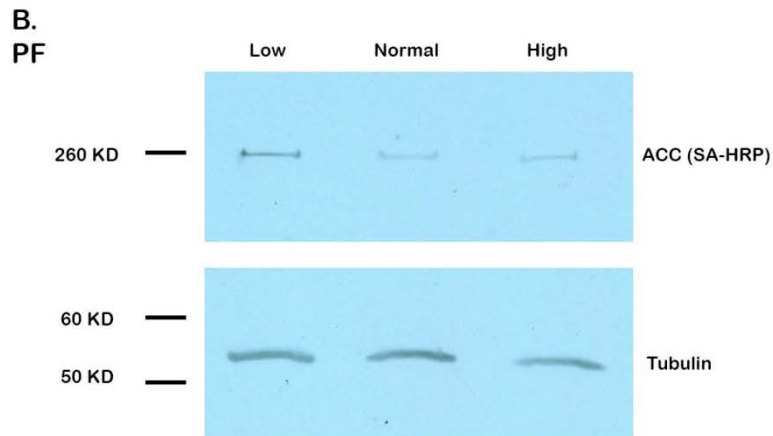
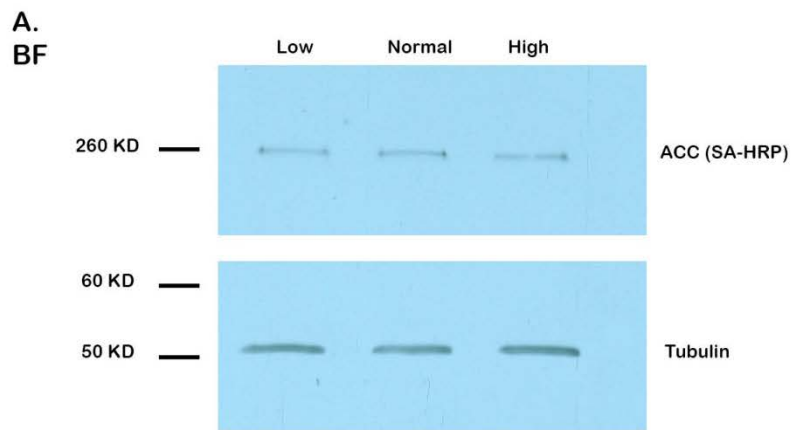


Figure 2.2: Environmental lipids affects TbACC protein levels in PF but not BF. BF (A.) and PF (B.) WT cells were grown in low, normal, and high lipid media to mid-logarithmic stage.

Lysates were prepared in the presence of HALT phosphatase inhibitor cocktail. 10 µg of total protein/lane was resolved by 10% SDS-PAGE and transferred to nitrocellulose. TbACC was detected by SA-HRP blotting (top panel) and the same blots were probed for tubulin as a loading control (bottom panel). Representative blots are shown in A and B. **(C.)** Densitometric quantification of three independent experiments. ACC signal was normalized to the tubulin loading control. Mean ± SEM is shown. ** P ≤ 0.01 for difference between PF cells grown in high lipid and low lipid media (Student's t-Test).

Effect of environmental lipids on TbACC activity

To examine the effect of environmental lipids on TbACC activity, lysates were prepared from cells grown in low, normal, and high lipid media and then assayed for ACC activity. TbACC enzyme activity was assayed by measuring the incorporation of [¹⁴C]CO₂ from radiolabeled [¹⁴C]NaHCO₃ in the presence of acetyl-CoA and ATP into the acid-resistant product [¹⁴C]malonyl-CoA, which was quantified by scintillation counting. In BFs, growth in high or low lipid media did not affect TbACC activity, as compared to growth in normal lipid media. In contrast, in PFs growth in high lipid media resulted in ~60% decrease in TbACC activity compared to growth in low lipid media (Fig. 2.3).

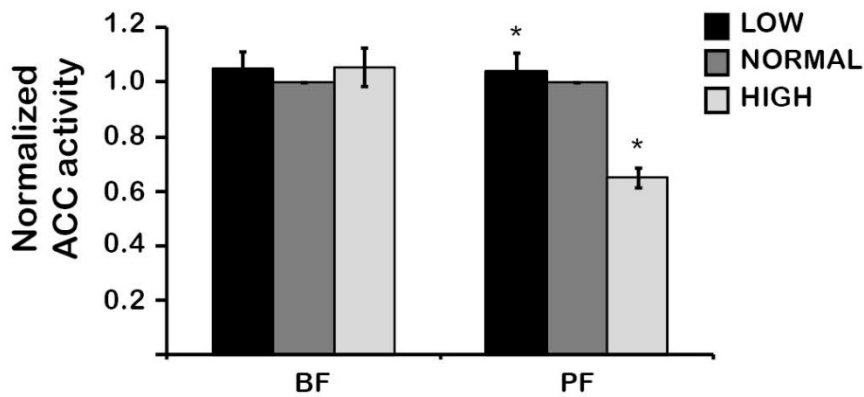


Figure 2.3: Environmental lipids affect TbACC activity in PF but not BF. BF and PF WT cells were grown in low, normal, and high lipid conditions to mid-logarithmic stage. Lysates were prepared in the presence of HALT phosphatase inhibitor cocktail, and equal amount of total protein was assayed for ACC activity by measuring incorporation of [^{14}C]NaHCO₃ into the acid-resistant malonyl-CoA product in the presence of ATP and acetyl-CoA. Values were first normalized to “no ATP” negative control before averaging. Average values were then expressed relative to that of normal media. Mean \pm SEM of 3 independent experiments is shown. ** $P \leq 0.01$ for difference between PF cells grown in high and low lipid conditions (Student’s t-Test).

Effect of exogenous C18:0 FA on TbACC activity

To confirm that the change in activity of TbACC in different environmental lipids was due to the lipid component of the media, we determined if addition of a single FA can affect TbACC activity. PFs were first grown in low lipid media to mid-logarithmic stage. The culture was then divided into 3 sub-cultures that were either maintained in low lipid media, or supplemented with serum to equal the high lipid media, or supplemented with 35 μM of stearate (C18:0) FA. ACC

enzyme activity was assayed as described above. PFs supplemented with serum (high lipid) or 35 μ M C18:0 FA showed a ~25% and ~40% decrease, respectively, in TbACC activity when compared to cells maintained in low lipid media (Fig. 2.4).

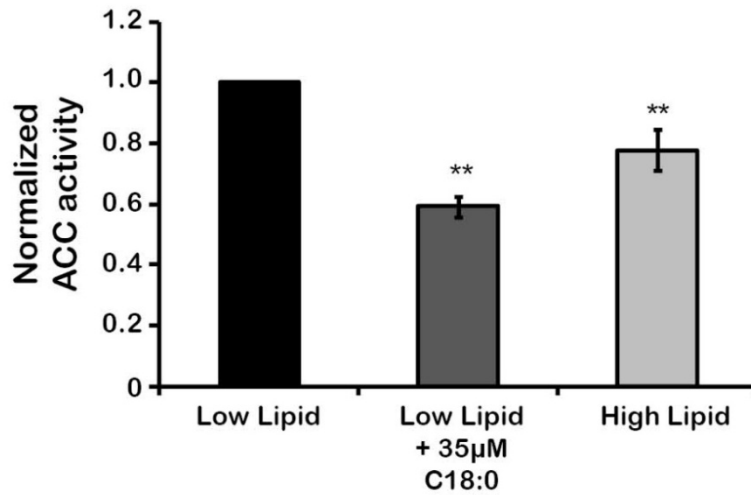


Figure 2.4: PF TbACC activity is reduced upon addition of exogenous C18:0 FA. PF WT cells were grown in low lipid conditions, then maintained in low lipid (Low Lipid) or supplemented with serum (High lipid) or 35 μ M C18:0 FA (Low Lipid + 35 μ M C18:0). Hypotonic lysates were assayed for ACC activity as described in Fig. 2.2. Values were normalized to the “no ATP” control before averaging, then averaged values expressed relative to the cells grown in low lipid conditions. Mean \pm SEM of 3 independent experiments is shown. ** $P \leq 0.01$ for difference from low lipid conditions (Student’s t-Test).

***In silico* analysis of potential phosphorylation sites in TbACC**

To determine if TbACC might be phosphorylated, the TbACC protein sequence (Tb 927.8.7100) was analyzed for potential serine, threonine, and

tyrosine phosphorylation sites using the NetPhos 2.0 prediction software (Bloom *et al.*, 1999). The NetPhos 2.0 output indicated the presence of 52 serine, 33 threonine, and 19 tyrosine potential phosphorylation sites with a confidence threshold ≥ 0.50 (Tables 2.1, 2.2, and 2.3). With a threshold of ≥ 0.90 , there were 19 phosphorylation sites, consisting of 13 serine, 3 threonine, and 3 tyrosine sites (Fig. 2.5.A and B).

To determine what kinases might potentially phosphorylate TbACC, KinasePhos 2.0 was used to assess the TbACC protein sequence for kinase-specific phosphorylation sites (Wong *et al.*, 2007). The output predicted the presence of a total of 29 kinase-specific phosphorylation sites with a confidence threshold >0.70 (Table 2.4) and included 20 protein kinase C (PKC) sites, 8 protein kinase A (PKA) sites, and 1 protein kinase B (PKB) site. Five of the sites predicted by the NetPhos 2.0 software were coincident with 3 predicted PKC sites and 2 predicted PKA with threshold of ≥ 0.90 . Interestingly, no phosphorylation sites specific to AMPK were predicted.

Table 2.1: Predicted phospho-serine sites

Position	Context	Score	Position	Context	Score
48	KGIDSVRSW	0.521	1197	KKSVSFLEH	0.994
147	FLGPSAKAM	0.717	1245	IRSDSTIKY	0.976
175	TVPWSGDEI	0.926	1279	QVGKSYKWR	0.993
199	KAYISTAAE	0.913	1313	VIVSSSSGA	0.816
396	PYDTSPIDF	0.918	1315	VSSSSGASA	0.909
430	FRPTSGRVE	0.994	1355	TSSDSVQEQ	0.992
441	AFKNSKECW	0.885	1370	VEPPSGCPA	0.899
469	GHIFSSAET	0.681	1397	SLLPSRGDE	0.993
553	LRMLSKRDE	0.997	1465	RFPLSRIPP	0.922
578	TEFLSNYES	0.990	1511	INPPSYIDS	0.996
600	MGLTSPTEI	0.995	1575	RIGLSAEVK	0.585
643	EKEPSSLRI	0.507	1600	YLVQSDYDE	0.908
644	KEPSSLRIS	0.925	1720	YSDNSQLGG	0.685
648	SLRISIGGK	0.989	1811	FDRDSWVES	0.995
705	PLRASTVGA	0.655	1815	SWVESLEGW	0.734
735	PDDPSKVAR	0.719	1858	ADPTSSEAF	0.974
766	ERLDSLARA	0.988	1874	WFPDSARKT	0.613
797	RRLKSAFSD	0.910	1902	WRGFSGGMR	0.610
827	RVVGS DHAT	0.747	1918	KFGASIVDN	0.890
921	VNLRSTGDG	0.929	1965	YCDGSARGG	0.873
958	LEEGSMMDL	0.932	1999	PRLRSLSPD	0.786
1046	ATAGSAENQ	0.945	2001	LRSLSPDHR	0.987
1084	SRCPSVCTV	0.852	2048	PWKDSRRRF	0.954
1143	FTYRSAHDW	0.991	2119	ELNVSTHNI	0.809
1160	VAPLSARRL	0.961	2125	HNIVSPTSA	0.887
1195	PKKKSVSFL	0.991	2128	VSPTSASAE	0.976

Table 2.2: Predicted phospho-threonine sites

Position	Context	Score
9	SPVTTMRPQ	0.941
60	HTGNTEAVE	0.601
70	TVMATPEDL	0.566
418	AVRVTAEDT	0.687
422	TAEDTDEGF	0.636
429	GFRPTSGRV	0.845
473	SSAETREEA	0.872
548	TAACLRML	0.665
688	VEEGTIVAE	0.817
730	VAEITPDDP	0.975
744	PREATEPWP	0.850
869	QVRETTGDT	0.922
870	VRETTGDTR	0.910
873	TTGDTRKVF	0.871
922	NLRSTGDGT	0.893
1079	NLQQTSRCP	0.909
1097	CRQFTEEEV	0.932
1140	PRTFTYRSA	0.978
1151	WREDTLIRN	0.930
1179	VMYPTPFKE	0.868
1190	VFHATPKKK	0.979
1210	RACVTPRDL	0.986
1246	RSDSTIKYP	0.604
1674	ISIVTGRSV	0.818
1694	RVIQTGDAP	0.700
1782	DRDVTYEPS	0.773
1825	KTVVTGRAT	0.823
1845	ETRPTRKCK	0.846
1857	PADPTSSEA	0.905
1878	SARKTADAL	0.821
2036	RMEATGVVR	0.778
2068	SLAATLVER	0.526
2179	LERTTAK	0.899

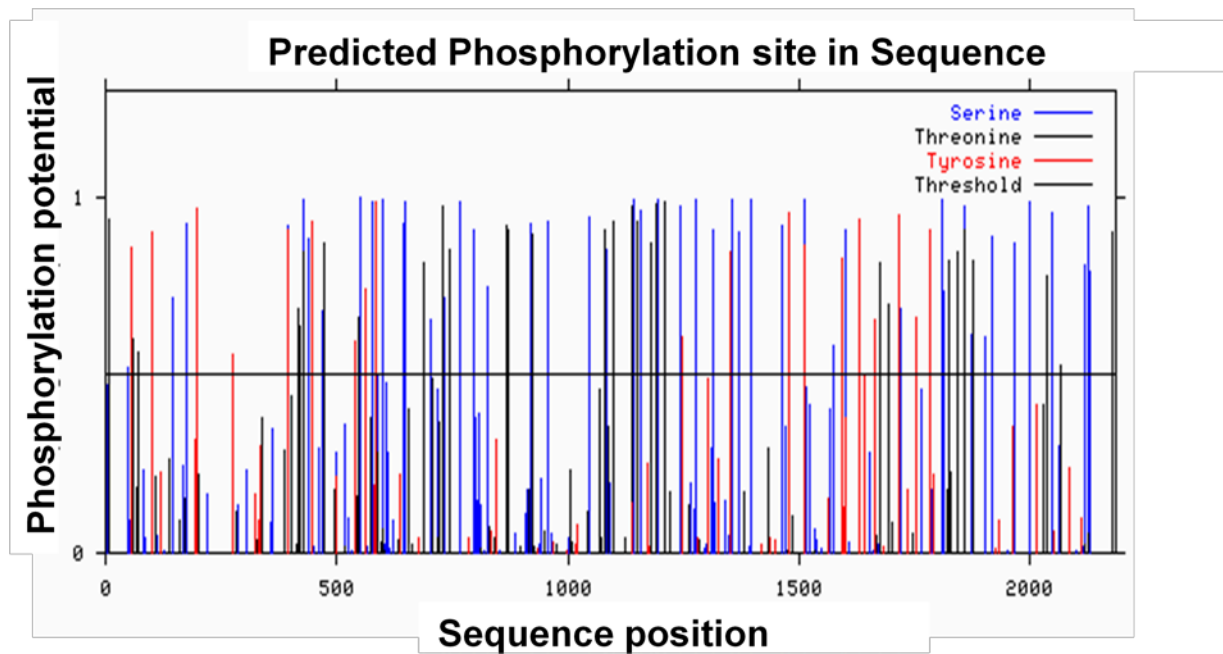
Table 2.3: Predicted phospho-tyrosine sites

Position	Context	Score
54	RSWLYVHTG	0.858
99	NRNNYANVD	0.904
197	YEKAYISTA	0.970
274	LADDYGDCI	0.561
393	GEQPYDTSP	0.907
447	ECWGYFSVG	0.934
540	QQDVYIALT	0.597
562	NHGRYVSFL	0.744
585	ESESYVNRS	0.985
1249	STIKYPKHN	0.607
1350	MNSLYTSSD	0.846
1478	ATELYLDPA	0.955
1512	NPPSYDSE	0.866
1594	EEAEYLYLV	0.827
1632	GEVRYVIRG	0.936
1664	MSKNYSNVP	0.658
1716	GKEVYSDNS	0.952
1754	RWLDYVPPV	0.665
1783	RDVTYEPSG	0.905

Table 2.4: Predicted kinase-specific phosphorylation sites

Site	Kinase	Score
T-8	PKC	0.71
T-108	PKC	0.71
S-166	PKC	0.71
T-338	PKC	0.89
T-401	PKC	0.78
S-531	PKC	0.72
T-744	PKB	0.76
S-797	PKA	0.77
S-912	PKA	0.77
S-939	PKA	0.74
T-1079	PKC	0.73
S-1084	PKA	0.71
T-1140	PKC	0.88
T-1190	PKC	0.80
S-1195	PKA	0.72
T-1246	PKC	0.90
S-1268	PKC	0.89

A.



B.

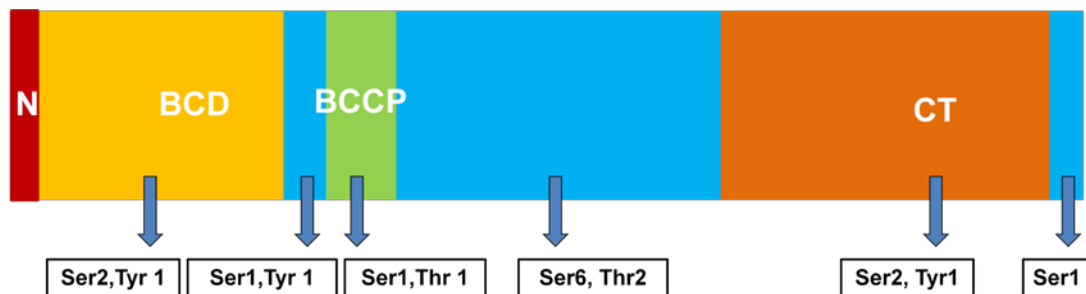
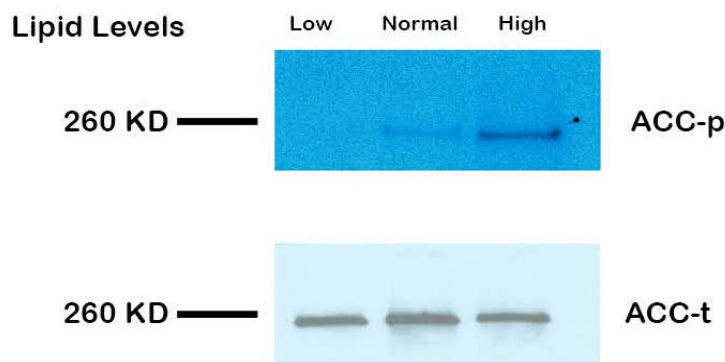


Figure 2.5: Phospho-prediction analysis. The TbACC predicted protein sequence was analyzed for potential phosphorylation sites using NetPhos 2.0 prediction software. **(A.)** Graph showing the frequency vs. predicted sites of phosphorylation. **(B.)** Cartoon showing high confidence (≥ 0.90) phosphorylation sites and their location in the TbACC protein. N, N-terminal domain; BCD, biotin carboxylase domain; BCCP, biotin carboxyl carrier protein domain; CT, carboxyl transferase domain; and C, C-terminal domain.

TbACC is a phosphoprotein whose phosphorylation is affected by environmental lipids

To confirm that TbACC is a phosphoprotein, *T. brucei* PF cells carrying a C-terminally myc-tagged ACC in one of the genomic loci (PF ACC-myc) were grown in low, normal, and high lipid media for 48 h, then metabolically labeled with [³²P]orthophosphate as the sole source of phosphate. [³²P]orthophosphate labeling of TbACC-myc immunoprecipitates confirmed that TbACC is phosphorylated in PFs (Fig. 2.6). Furthermore, TbACC-myc showed a 4-fold increase in phosphorylation in high lipid media compared to normal lipid media, while TbACC-myc showed an 80% decrease in phosphorylation in low lipid media compared to normal media (Fig 2.6.A and B).

A.



B.

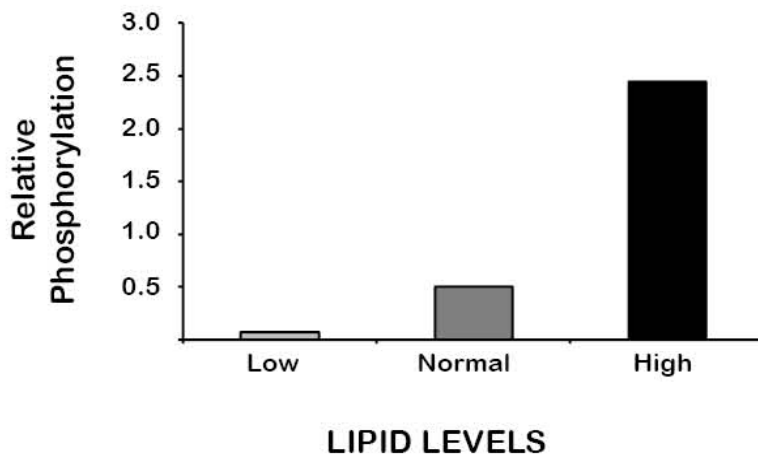


Figure 2.6: Effect of environmental lipids on PF TbACC phosphorylation via

[³²P]phosphate labeling. PF ACC-myc cells were grown in normal lipid media to mid-logarithmic phase. Cells were harvested and incubated with 1-2 mCi [³²P]orthophosphate in low, normal, and high lipid phosphate-free media for 16 h. TbACC-myc immunoprecipitates were resolved by SDS-PAGE, transferred on nitrocellulose, and blots assessed by autoradiography (**A.**, top panel).

Identically-loaded blots were prepared in parallel and probed for total TbACC by SA-HRP blotting (**A.**, bottom panel) (**B.**) Densitometric analysis of the blots in **A.** TbACC-myc phosphorylation values were normalized to total TbACC loaded.

We further confirmed TbACC phosphorylation using a non-radioactive phospho-staining method. PF ACC-myc cells were grown in low, normal, and high lipid media, and TbACC-myc immunoprecipitates were resolved by SDS-PAGE and stained for phosphoproteins using a non-specific phosphoprotein gel stain, Pro Diamond Q. TbACC-myc purified from the low, normal, and high lipid growth condition showed different levels of phosphorylation (Fig. 2.7A and B). Low lipid media resulted in a 50% decrease in TbACC phosphorylation compared to normal lipid media. In contrast, high lipid media induced the highest level of TbACC phosphorylation with an increase of 2.5-fold over that in normal lipid conditions. Similar experiments performed in BF ACC-myc cells indicated that phosphorylation of TbACC was not detected in BFs (Fig. 2.8).

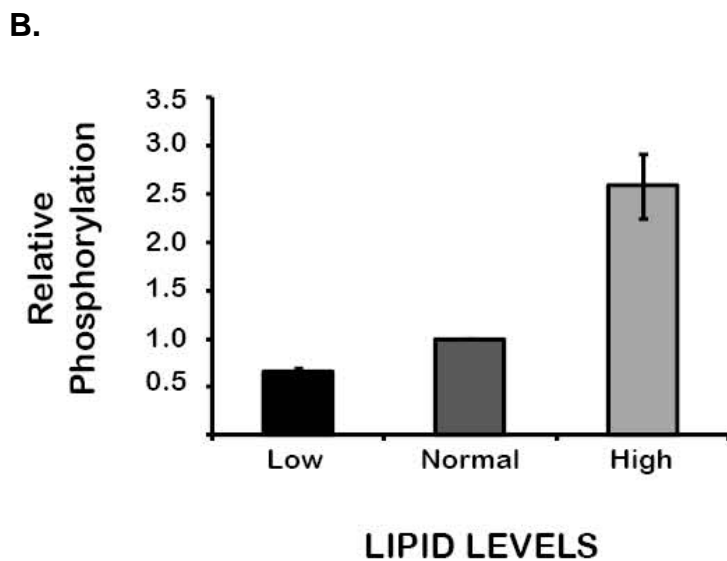
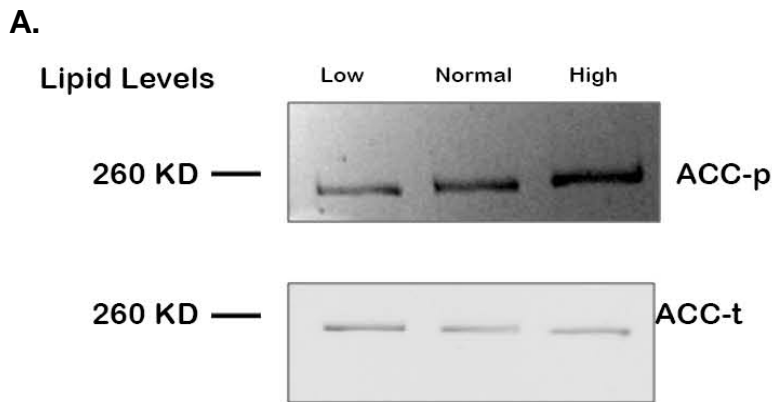


Figure 2.7: Effect of environmental lipids on PF TbACC phosphorylation via phosphoprotein gel staining. PF TbACC-myc immunoprecipitates from cells grown in low, normal, and high lipid media were prepared as described for Fig. 2.6, resolved by SDS-PAGE, and the gels stained with Pro Diamond Q phosphoprotein gel stain and imaged under UV (**A.**, upper panel). Identically-loaded gels prepared in parallel were transferred to nitrocellulose and the blots probed for total TbACC by SA-HRP blotting (**A.**, lower panel). (**B.**) Densitometric analysis of 3 independent experiments. PF TbACC-myc phosphorylation values (ACC-p) were normalized to total TbACC-myc loaded (ACC-t). Mean \pm SEM is shown. *, $P \leq 0.01$ for differences between high lipid and low lipid conditions (Student's t-Test).

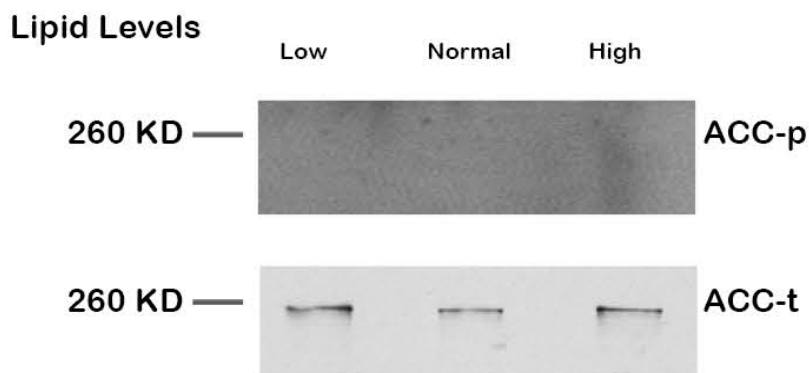


Figure 2.8: Effect of environmental lipids on BF TbACC phosphorylation via phosphoprotein gel staining. BF TbACC-myc immunoprecipitates from cells grown in low, normal, and high lipid media were analyzed by Pro Diamond Q phosphoprotein gel staining as described for Fig. 2.6, except 5×10^8 cells were used. Upper panel, UV imaging of phosphoprotein gel staining of TbACC (ACC-p). Lower panel, SA-HRP blotting for total TbACC (ACC-t) of identically-loaded blots processed in parallel.

Phosphorylation reduces the activity of TbACC-myc

To determine the effect of phosphorylation on TbACC activity, PF TbACC-myc cells were grown in high lipid media and TbACC-myc isolated by pull-down with anti-myc sepharose beads. TbACC-myc-bound beads were treated with lambda phosphatase or left untreated as a control. The TbACC activity of the TbACC-myc bound to the beads was assayed as described previously. To confirm successful dephosphorylation, TbACC-myc pull-downs were also assessed by SDS-PAGE and Pro Diamond Q phosphoprotein gel staining as described previously. Phosphoprotein staining confirmed successful

dephosphorylation of TbACC-myc by lambda phosphatase treatment (Fig. 2.9A). Dephosphorylation of TbACC-myc resulted in a 2-fold increase in TbACC-myc activity as compared to the untreated control, indicating that phosphorylation is an inhibitory modification (Fig. 2.9B).

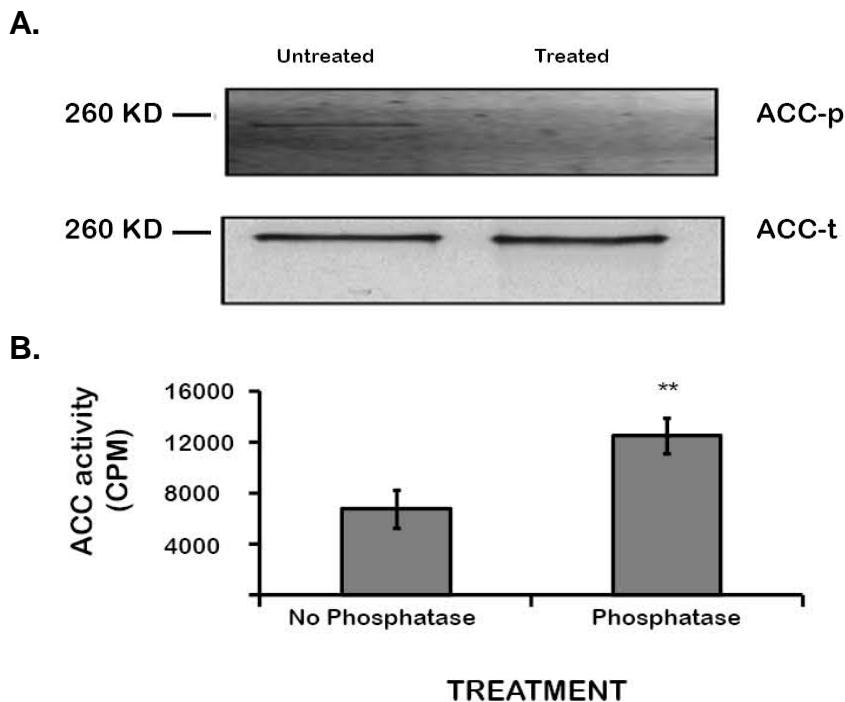


Figure 2.9: Phosphorylation of TbACC reduces activity. PF TbACC-myc cells were grown in normal media to mid-log phase. TbACC-myc was pulled down by anti-myc sepharose beads and directly treated with 400 U of lambda phosphatase or mock treated as a control. TbACC-myc pull-downs were assessed for phosphorylation by SDS-PAGE and phosphoprotein staining. **(A.)** Representative UV image of phosphoprotein gel staining (upper panel) and SA-HRP blotting of TbACC-myc pull-downs processed in parallel (lower panel). **(B.)** Phosphatase and mock-treated TbACC-myc pull-downs were assayed for ACC activity. Values were normalized to “no ATP” control. Mean \pm SEM of 3 independent experiments are shown. *, $P \leq 0.01$ for difference between phosphatase-treated and untreated (Student’s t-Test).

DISCUSSION

It is well documented that *T. brucei* can readily take up and use lipids, including FAs, from its environment (Dixon *et al.*, 1971; Voorheis, 1980; Bowes *et al.*, 1993; Coppens *et al.*, 1995; Lee *et al.*, 1999). Given that *T. brucei* can satisfy its FA requirements either through uptake from the host or through *de novo* synthesis, the parasite may have a mechanism to modulate FA synthesis in response to environmental lipid availability. We hypothesized that TbACC serves as a regulatory control point and thus, is regulated by environmental lipids. As serum is the sole source of lipid in the media, we determined the effect of modulating the serum lipids in the growth media on TbACC expression and activity.

Quantitative RT-PCR analysis of TbACC mRNA levels showed no significant difference when BF and PF *T. brucei* were grown in low, normal, or high lipid media. Thus, TbACC does not appear to be transcriptionally regulated. This result is consistent with studies that indicate minimal transcriptional regulation in *T. brucei*, due to their polycistronic mode of transcription and the absence of classical RNA Pol II promoters (Campbell *et al.*, 2003; Palenchar and Bellofatto, 2006; Günzl *et al.*, 2007; Martinez-Calvillo *et al.*, 2010).

We next looked at TbACC protein levels in cells grown in low, normal, and high lipid media by western blot analysis. In BFs, there was no significant difference in TbACC protein levels. However, in PFs we observed the highest levels of TbACC protein in the low lipid media and the lowest level in the high

lipid media. Growth in low, normal, or high lipid media had no effect on TbACC enzyme activity in BFs, but in PFs, TbACC was highest in the low lipid media and lowest in the high lipid media. The reduction of TbACC activity seen when low lipid media was supplemented with C18:0 FA confirms the idea that the regulation of PF TbACC is due to the lipid component of the media. Together, these results demonstrate that TbACC is regulated post-transcriptionally in PFs in response to environmental lipids, both at the level of protein and activity. TbACC protein levels may be regulated due to protein degradation in high lipid conditions. Control at the level of protein stability occurs due to translational and post translational regulation in Trypanosomes (Torri *et al.*, 1993; Gale *et al.*, 1994). Proteasomal mediated degradation has already been established in *T.brucei* (Li *et al.*, 2002). This selective degradation can be due to environmental sensing and post translational modification of TbACC resulting in targeted TbACC degradation in high lipid environment.

In other organisms, it is well documented that ACC activity is regulated through phosphorylation by AMPK and PKA (Hardie *et al.*, 1992; Brownsey *et al.*, 2006). Metabolic labeling experiments demonstrated the presence of at least four hormonal-responsive phosphorylation sites in mammalian ACCs (Brownsey *et al.*, 1992; Hardie *et al.*, 1992). Glucagon treatment or high cellular AMP levels lead to the rapid phosphorylation and inactivation of ACC. Because ACC is regulated by phosphorylation in all organisms studied to date (Kim, 1983; Kim *et al.*, 1989), we hypothesized that TbACC is also regulated by phosphorylation.

First, *in silico* analysis of the TbACC protein sequence for potential phosphorylation sites and kinase-specific sites revealed multiple predicted high-confidence sites, including consensus sites for PKA, PKC, and PKB kinases, homologs for which are present in the *T. brucei* genome (Nett *et al.*, 2009). However, none of these predicted phosphorylation sites corresponded with known, highly conserved, phosphorylation sites in human and yeast ACCs (Kimberly Paul, personal communication). Interestingly, no sites for the best-characterized ACC kinase, AMPK, were predicted despite the fact that AMPK is present in *T. brucei* (Clemmens *et al.*, 2009). Although a negative observation and thus inconclusive, the sole phosphoproteomic study in *T. brucei* available at the time we initiated these studies showed no evidence that TbACC was phosphorylated (Nett *et al.*, 2009).

Metabolic labeling with [³²P]orthophosphate and phosphoprotein staining of PFs confirmed that TbACC is phosphorylated in PF *T. brucei*, consistent with the detection of ACC phosphorylation in other species by ³²P-labeling (Witters, 1981; Thampi and Wakil, 1985). Further, these studies revealed that TbACC is differentially phosphorylated in PFs in response to environmental lipids. The relative phosphorylation level of TbACC increased in the presence of high environmental lipid levels and decreased as the environmental lipid levels decreased. This is consistent with the FA induction of the AMPK signaling cascade in response to environmental lipids observed in muscle (Li *et al.*, 2013). We also demonstrated that dephosphorylation of TbACC leads to an increase in

its activity, indicating that phosphorylation is an inactivating post-translational modification of TbACC. These observations on TbACC phosphorylation are consistent with work on ACC regulation in humans and yeast, which demonstrated that treatment of cells with starvation-stimulated hormones such as glucagon leads to rapid phosphorylation and inactivation of ACC (Hardie *et al.*, 1992). Likewise, dephosphorylation of chicken liver ACC by treating it with protein phosphatase caused a 5-fold increase in ACC activity (Wada and Tanabe, 1983).

We propose that when PFs have an adequate supply of lipid in their environment, they rely on uptake of exogenous fatty acids from the media and correspondingly phosphorylate and downregulate TbACC. However, under limiting lipid conditions, the parasite dephosphorylates TbACC, thereby activating it in order to synthesize its own FAs. This idea is consistent with the general upregulation of FA synthesis seen when PFs were grown in low lipid media (Lee *et al.*, 2006) and studies showing that RNAi of TbACC or another FA synthesis enzyme enoyl-CoA reductase had no effect on growth in normal or high lipid media, but reduced growth only in low lipid media (Lee *et al.*, 2006; Vigueira *et al.*, 2011).

The phosphorylation of human ACC isoforms is complex, involving several phosphorylation sites, thus the impact of a specific phosphorylation state and catalytic activity has been controversial (Lane *et al.*, 1974; Kim, 1979; Tipper and Witters, 1982; Tipper *et al.*, 1983). Given the number of potential phosphorylation

sites in TbACC, phosphoregulation of TbACC may also be subject to complex regulation by multiple kinases. Supporting this idea, a recent phosphoproteomic study in *T. brucei* (Urbaniak *et al.*, 2013) identified three TbACC phosphopeptides, corresponding to Ser5, Ser1999, and Ser2001. The latter two phosphoserine sites were predicted by NetPhos 2.0 as potential phosphorylation sites with confidence scores of 0.786 and 0.987, respectively. None of the three phosphoserine sites were predicted by NetPhosK 1.0 to be a kinase-specific site, possibly owing to the divergence of consensus sites between *T. brucei* kinases and other eukaryotic kinases (Brownsey *et al.*, 1992; Hardie *et al.*, 1992). Future work will be needed to determine the identity of the TbACC kinase(s) and the role of each phosphorylation site.

In contrast to PFs, we observed no changes in TbACC protein levels or activity in BFs grown in different lipid conditions. In addition, we observed no phospholabeling of TbACC in BFs grown in low, normal, or high lipid media. These data suggest that BF TbACC does not appear to be regulated by environmental lipids and that under typical culture conditions, BF TbACC is constitutively dephosphorylated and active. We have demonstrated, however, that phosphorylated TbACC can be detected in BFs, when the cells were treated with a compound, epigallocatechin gallate, which is a known activator of AMPK (Vigueira *et al.*, 2012).

Constitutive activation of TbACC in BFs can have several possible explanations. First, there may be a difference in the sensitivity of BFs to

environmental lipids compared to PFs. Perhaps the altered lipid levels in the low or high lipid media did not achieve the threshold needed for BFs to modulate their TbACC expression or activity. We attempted to further lower the lipid in media by delipidation or reduction of serum, but the resulting media did not support *T. brucei* growth.

Second, there may be a high demand of FAs in BFs due to the VSG coat, which plays a vital role in immune evasion in *T. brucei* (Donelson, 2003). The VSG GPI-anchors contain exclusively myristate, a relatively scarce FA in blood (Paul *et al.*, 2001), and at any given time, a single parasite expresses around a 10 million copies of VSG, each of them anchored by two myristates (Ferguson and Cross, 1984). Thus, BF *T. brucei* may rely on TbACC and ongoing FA synthesis to make enough myristates to maintain the VSG coat.

Third, constitutive activation of TbACC and ongoing FA synthesis may be required to support a second immune evasion tactic in BF *T. brucei*, the upregulation of endocytosis (Field and Carrington, 2009). The rate of endocytosis in BFs is 10-fold higher than that of PFs (Natesan, *et al.*, 2007), and this high rate of endocytosis is thought to enable BF *T. brucei* to clear surface-bound antibodies and evade complement-mediated lysis (Balber *et al.*, 1979; Ferrante and Allison, 1983; Donelson *et al.*, 1998). Clearance of antibody from the surface is achieved by internalization of VSGs bound to antibodies into a recycling endocytic pathway, where after sorting, the antibody is degraded and VSG is recycled back to the surface (Donelson *et al.*, 1999; Field and Carrington, 2009).

The high rate of membrane turnover needed to sustain this rapid rate of endocytosis and recycling may require a high rate of TbACC activity and FA synthesis in BFs in order to maintain membrane homeostasis. Studies from our lab provide support for the idea that activation of TbACC is required to sustain high rates of endocytosis (Mcknight, 2012). RNAi of ACC in BF *T. brucei* resulted in a reduction in both fluid phase and receptor-mediated endocytosis, slowed kinetics of antibody clearance, and increased susceptibility to killing by complement-mediated lysis (McKnight, 2012).

In summary, the work presented in this chapter demonstrate that TbACC is regulated by environmental lipids in PFs but not in BFs. We showed that *T. brucei* regulates its FA metabolism so that FA synthesis is down-regulated in lipid-rich environments and is upregulated when environmental lipids are limiting. These results also suggest that *T. brucei* can transduce its environmental lipid status into regulatory signals that not only alter TbACC protein levels but also its activity, suggesting multiple post-transcriptional regulatory mechanisms. Finally, we have demonstrated that one such environmental regulatory mechanism is phosphorylation, which occurs under high lipid conditions and serves to reduce TbACC activity.

MATERIALS AND METHODS

Reagents

All chemicals and reagents used in this study were purchased from Thermo Fisher Scientific and Sigma except: Iscove's Modified Dulbecco's Medium (IMDM) and Minimum Essential Media (MEM) (Invitrogen), Serum Plus (JRH Biosciences), and delipidated Fetal Bovine Serum (Cocalico Biologicals).

***T. brucei* strains and growth media**

Wild-type PF and BF *T. brucei* strain 427 were a kind gift from Dr. Paul Englund (Johns Hopkins School of Medicine). ACC-myc PF cell line expressing one genomic loci of ACC with a C-terminal myc epitope tag was developed by Dr. Patrick Vigueira (Vigueira *et al.*, 2011). A BF ACC-myc cell line was generated using the same method as in (Vigueira *et al.*, 2011). Correct integration of myc epitope tag was verified using diagnostic PCR and western blotting.

Serum is the only source of lipids in the *T. brucei* growth media. Three different sera were used to prepare media: Fetal Bovine Serum (FBS), delipidated Fetal Bovine Serum (dFBS), and Serum Plus (SP) (see Table 2.5 for media formulations). According to the manufacturers, both dFBS and SP have 20% of the lipids found in FBS. The normal growth medium for PFs is SDM-79 (Brun and Shonenberger, 1979) supplemented with heat-inactivated 10% FBS, making the medium 10% total serum lipids. For BFs, the normal growth medium

is HMI-9 (Hirumi and Hirumi, 1989) supplemented with heat-inactivated 10% FBS and 10% SP, making the medium 12% total serum lipids.

To prepare low lipid media for PFs, heat-inactivated 10% dFBS replaced the FBS giving a final concentration of 2% total serum lipids. BF low lipid media was prepared with 10% heat-inactivated dFBS and 10% SP, giving a final concentration of 4% total serum lipids. For both BFs and PFs, high lipid media was prepared by doubling the heat- inactivated FBS, resulting in a final of 20% and 22% total serum lipids for BF and PF media, respectively.

TABLE 2.5: Formula for low, normal, and high lipid *T. brucei* growth media

	Condition	Delipidated Serum	Fetal Bovine Serum	Serum Plus	Serum lipid Equivalents
BSF	Low	10%	0%	10%	4% (~0.2X)
	Normal	_____	10%	10%	12% (1X)
	High	_____	20%	10%	22% ~(2X)
PCF	Low	10%	0%	_____	2% (0.2X)
	Normal	_____	10%	_____	10% (1X)
	High	_____	20%	_____	20% (2X)

Lysate preparation

Hypotonic lysates were prepared from 5×10^8 cells as described (Morita *et al.*, 2000) or from 1×10^9 cells as described (Bangs 1993; Roggy and Bangs,

1999). For both lysis methods, a protease inhibitor cocktail (1 µg/ml Leupeptin, 0.1 mM tosyllysine chloromethyl ketone hydrochloride (TLCK)) and HALT phosphatase inhibitor cocktail (Thermo Scientific) were added during the lysis. Hypotonic lysates were sheared by passage through a 27^{1/2} gauge needle three times, and lysates were clarified by centrifugation at 4 °C at 1,000 x g (10 min.) followed by 16,000 x g (10 min.). RIPA (radioimmunoprecipitate assay) lysates were prepared from 1 x 10⁹ cells/ml as described (Vigueira *et al.*, 2011). Cell lysates were centrifuged for 30 min. at 16,000 x g at 4 °C to remove cellular debris.

Quantitative reverse transcriptase PCR

BF and PF WT cells (3-5 x 10⁶) were grown to mid-logarithmic stage and washed in bicine-buffered saline glucose buffer (Raper *et al.*, 1993). Total RNA was extracted using Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's protocol, except RNA was eluted using 40 µl of elution buffer. *T. brucei* ACC-specific primers were designed using Primer3 (Rozen and Skaletsky, 2000): Forward ACC primer, 5'-TTT CGT AAG TTG AAG TCT GG-3'; and reverse ACC primer, 5'-CTT AGT CGG ATC AAT GTC AC-3'. Actin was used as a control and actin-specific primers were a kind gift from Dr. Meredith Morris (Clemson University): forward Actin primer, 5'-GCC ACG TAT TTC CAT CCA TC-3'; and reverse Actin primer, 5'-CCT GAG CTT CAT CAC CAA CA-3'. cDNA synthesis was performed on 100-200 ng total RNA using the iScript cDNA

Synthesis Kit (Bio-rad) using the manufacturer's protocol. The resultant cDNA was used as template with 0.2 μ M of each primer pair and the SensiFAST SYBR & Fluorescein Mix (Bioline) in quantitative PCR with 1 cycle at 95 °C for 2 min, and then 40 cycles as follows: 95 °C for 30 s., 57 °C for 1 min., and 72 °C for 30 s. β -actin served as the endogenous control. $\Delta\Delta$ Ct method was used to calculate the relative expression levels.

Western blotting

Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. For streptavidin blotting, the membrane was blocked in 2.5% nonfat dry milk, 1X Tris Buffered Saline (TBS), 0.05% Tween 20 for \geq 1 h. and washed three times (5 min. each) in streptavidin wash buffer (0.2% nonfat dry milk, 1X TBS, 0.05% Tween 20). To probe for ACC, blots were probed with 1:200 SA-HRP (Pierce), which recognizes the biotin prosthetic group (Nikolau *et al.*, 1985) for \geq 2 h. The blots were then washed twice with streptavidin wash buffer and twice with 1X TBS, 0.05% Tween 20 before development with Super Signal West Pico Chemiluminescent substrate kit (Thermo Scientific).

For anti-tubulin blotting, membranes were blocked in 2.5% nonfat dry milk, 1X TBS, 0.05% Tween 20 for \geq 1 hour then probed with mouse anti-tubulin (Pierce) diluted 1:50,000 in blocking buffer. The blots were washed thrice in blocking buffer and then probed with peroxidase-conjugated goat anti-mouse IgG (Rockland) diluted 1:10,000 in blocking buffer. Blots were then washed twice with

blocking buffer and twice with 1XTBS, 0.05% Tween 20 before chemiluminescent development.

For anti-c-myc blotting, membranes were blocked in 5% nonfat dry milk, 1X TBS, 0.05% Tween 20 for ≥ 1 h., then probed for myc-tagged ACC using mouse anti-c-myc monoclonal antibodies (sc 40, Santa Cruz Biotechnology) diluted 1:250 in blocking buffer. The blots were washed twice in blocking buffer and then probed with peroxidase-conjugated goat anti-mouse IgG (Rockland) diluted at 1:10,000 in blocking buffer. Blots were then washed twice with blocking buffer and twice with 1X TBS, 0.05% Tween 20 before chemiluminescent development. Appropriately exposed blots were analyzed by densitometry using Image J software (NIH) and values normalized to the indicated loading control.

ACC activity assay

PF and BF cells were grown to logarithmic stage ($1-2.5 \times 10^7$ PF cells/ml and $2.5-5 \times 10^6$ BF cells/ml) in low, normal and high lipid media. Hypotonic lysates were prepared in the presence of 1X HALT phosphatase inhibitor cocktail (Thermo Scientific). To remove endogenous CoA substrates and ATP, lysates were fractionated through a 1.5-2 ml G-50 sephadex column equilibrated in biotin carboxylase buffer (50 mM Tris-Cl pH 8.0, 5 mM $MgCl_2$, 5 mM DTT) supplemented with 1X HALT phosphatase inhibitor cocktail. The protein concentration of the chromatographed lysates were determined using the Bradford protein assay kit (Bio-Rad) using BSA as a standard. Equal protein was

assayed for ACC activity as described (Vigueira *et al.*, 2011). ACC activity was normalized to the background (no ATP) and expressed as a percent of ACC activity in normal lipid media. Statistical significance of differences was assessed using Student's T-test (Microsoft Excel).

TbACC-myc immunoprecipitation

TbACC-myc was purified from hypotonic PF and BF lysates using Pierce c-myc-Tag IP/Co-IP kit (Thermo-Fischer). The manufacturer's protocol was modified as follows to obtain the maximum yield: hypotonic lysates (500 μ l or 5 x 10⁸ cell equivalents) were incubated with 10-15 μ l of anti-myc agarose beads overnight at 4 °C with constant end-over-end mixing. Then the lysate/beads mixture was loaded into an empty spin column and unbound proteins removed with 3 washes with 1X TBS, 0.5% Tween 20. Bound TbACC-myc was eluted using 150 mM glycine pH 2.8, added 10 μ l at a time. Elution volumes were 100 μ l and 60 μ l for PF and BF TbACC-myc, respectively. Eluted TbACC-myc was immediately neutralized by the addition of 20 μ l (PF) and 10 μ l buffer (BF) of 100 mM Tris-Cl pH 9.5).

Metabolic [³²P]labeling of TbACC-myc

PF ACC-myc cells were grown in normal medium to mid-logarithmic phase (1-2.5 x 10⁷ cells/ml). The cells were harvested by centrifugation and washed 3X in Cunningham's phosphate-free medium (Kaminsky *et al.*, 1983). Cells were

resuspended to a final concentration 0.5×10^8 cells/ml in Cunningham's phosphate-free medium supplemented with dialyzed serum to generate low, normal, and high lipid media (Table 2.1). Cells were then incubated with 1-2 mCi [^{32}P]orthophosphate (ARC # 0103, 350 mCi/ml) for 16 h. at 28 °C/ 5% CO_2 . As a control, cells were incubated with the equivalent concentration of non-radioactive phosphate (10 mM KPi, pH 7.0). After labeling, the cells were harvested, hypotonic lysates prepared in the presence of HALT phosphatase inhibitor cocktail, and TbACC-myc purified by immunoprecipitation. TbACC-myc immunoprecipitates were resolved using SDS-PAGE and transferred to nitrocellulose. TbACC-myc blots were first assessed by autoradiography by exposing it to X-ray film for 10 days at room temperature. The same blot was then blotted using SA-HRP for total ACC as a loading control. Autoradiographs and blots were quantified by densitometry using ImageJ software (NIH) and values normalized using the loading control.

Phosphoprotein gel staining

Tb ACC-myc immunoprecipitates from hypotonic lysates of PF TbACC-myc cells grown in low, normal, and high lipid media were resolved by SDS-PAGE, and the gels were processed and stained with Pro Diamond Q Phosphoprotein Gel stain (Invitrogen) according to the manufacturer's directions. Phosphostained gels were imaged under UV using the Gel Doc XR Imaging System (Bio-Rad). In parallel, an identically-loaded non-stained gel was

transferred to nitrocellulose and probed with SA-HRP to detect total ACC loaded. Appropriately exposed blots and gel images were analyzed by densitometry using Image J software (NIH). Values for phosphorylated ACC-myc were normalized to total ACC loaded.

Lambda phosphatase treatment

Hypotonic lysates prepared from PF TbACC-myc cells grown to log-phase in normal lipid media were incubated with the Pierce ProFound c-Myc Tag IP/Co-IP kit (Thermo Fischer) for 16h at 4 °C with constant end-to-end mixing. TbACC-myc bound beads were washed 5X with 500 µl BC buffer then treated with 400 U of Lambda phosphatase (NEB) in a final volume of 200 µl in the provided buffer according to the manufacturer's protocol. As a control, TbACC-myc bound beads were incubated as above, but Lambda phosphatase was omitted. Treated or control TbACC-myc bound beads were then washed 2X in BC buffer and assayed for ACC activity as described above by adding assay components directly to TbACC bound beads.

Phosphatase treated and control TbACC-myc immunoprecipitates were resolved by SDS-PAGE and the gels stained with Pro Diamond Q phosphostain. In parallel, an identically-loaded non-stained gel was transferred to nitrocellulose and probed with SA-HRP to detect total ACC loaded. Appropriately exposed blots and gel images were analyzed by densitometry using Image J software

(NIH). Values for phosphorylated TbACC-myc were normalized to total ACC loaded.

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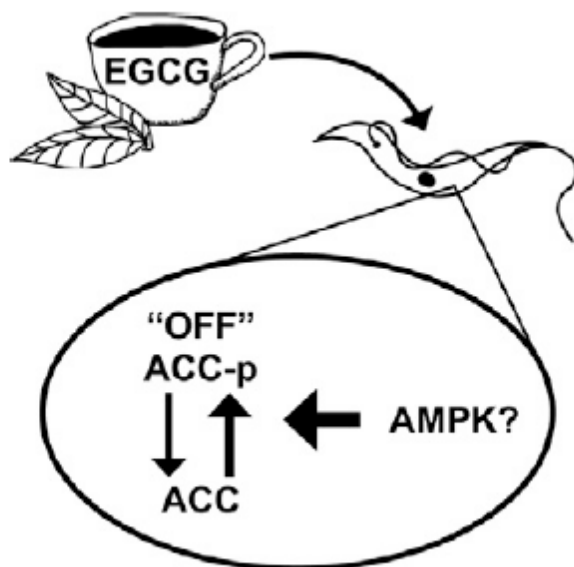
CHAPTER 3

EFFECTS OF THE GREEN TEA CATECHIN (-)-EPIGALLOCATECHIN

GALLATE ON *Trypanosoma brucei*

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ABSTRACT

The current pharmacopeia to treat the lethal human and animal diseases caused by the protozoan parasite *Trypanosoma brucei* remains limited. The parasite's ability to undergo antigenic variation represents a considerable barrier to vaccine development, making the identification of new drug targets extremely important. Recent studies have demonstrated that fatty acid synthesis is important for growth and virulence of *T. brucei brucei*, suggesting this pathway may have

therapeutic potential. The first committed step of fatty acid synthesis is catalyzed by acetyl-CoA carboxylase (ACC), which is a known target of (-)-epigallocatechin-3-gallate (EGCG), an active polyphenol compound found in green tea. EGCG exerts its effects on ACC through activation of AMP-dependent protein kinase, which phosphorylates and inhibits ACC. We found that EGCG inhibited TbACC activity with an EC₅₀ of 37 μM and 55 μM for bloodstream form and procyclic form lysates, respectively. Treatment with 100 μM EGCG induced a 4.7- and 1.7- fold increase in TbACC phosphorylation in bloodstream form and procyclic lysates. EGCG also inhibited the growth of bloodstream and procyclic parasites in culture, with a 48 h EC₅₀ of 33 μM and 27 μM, respectively, which is greater than the EGCG plasma levels typically achievable in humans through oral dosing. Daily intraperitoneal administration of EGCG did not reduce the virulence of an acute mouse model of *T. b. brucei* infection. These data suggest a reduced potential for EGCG to treat *T. brucei* infections, but suggest that EGCG may prove to be useful as a tool to probe ACC regulation.

INTRODUCTION

The protozoan parasite *Trypanosoma brucei* (sub-species *gambiense* and *rhodesiense*) are the causative agent of African sleeping sickness, a fatal human disease that ranges across sub-Saharan Africa. In addition to causing substantial morbidity and mortality in humans, a third sub-species, *T. brucei brucei* is responsible for causing nagana, a livestock disease that results in wasting and

death. Nagana imposes a tremendous economic burden on the region, causing 4.5 billion dollars in economic losses each year (FAO, 2007). Vaccine development is confounded by the parasite's ability to switch its surface coat through antigenic variation (Horn and McCulloch, 2010). Chemotherapeutics are relied upon to battle the disease, yet currently approved drugs cause undesirable side effects, are subject to failure, and can be too expensive for citizens of economically depressed regions (Castillo *et al.*, 2010). To meet this urgent need, investigation of existing compounds are an important avenue to identify potential new drugs that are effective, safe, and economical.

Green tea is amongst the most widely consumed beverages worldwide and is often touted for its wealth of medicinal effects (Moon *et al.*, 2007; Khan and Mukhtar, 2008; Thielecke and Boschmann, 2009; Ahmed, 2010). The best-studied active components of green tea are the catechins, of which (-)-epigallocatechin-3-gallate (EGCG) is one of the most abundant (Lin *et al.*, 2003). In addition to numerous other pathways, EGCG has been demonstrated to inhibit fatty acid synthesis (Wang and Tian, 2001; Brusselmans *et al.*, 2003) through its effect on the regulation of acetyl-CoA carboxylase (ACC) (Huang *et al.*, 2009). ACC catalyzes the first committed step in fatty acid synthesis: the ATP-dependent carboxylation of acetyl-CoA, which provides the two-carbon donor, malonyl-CoA, for fatty acid synthesis (Tong and Harwood, 2006). ACC is negatively regulated by phosphorylation by AMP-activated protein kinase (AMPK), a key regulator of cellular energy metabolism (Barber *et al.*, 2005;

Brownsey *et al.*, 2006). EGCG treatment activates AMPK, leading to increased phosphorylation of human ACC, which resulted in its inhibition (Moon *et al.*, 2007; Huang *et al.*, 2009).

In *T. b. brucei*, both the cytoplasmic and mitochondrial fatty acid synthesis pathways are important for growth in culture and virulence in mouse models (Lee *et al.*, 2006; Lee *et al.*, 2007; Stephens *et al.*, 2007; Vigueira and Paul, 2011). We have recently demonstrated that RNA interference (RNAi)-mediated gene knock-down of ACC in *T. b. brucei* reduced fatty acid elongation activity in intact cells and reduced virulence in a mouse model of infection (Vigueira and Paul, 2011). In addition, treatment with thiolactomycin, an inhibitor of fatty acid synthetase, inhibited fatty acid synthesis activity and growth of *T. b. brucei* in culture (Morita *et al.*, 2000). Taken together, these results suggest that fatty acid synthesis has the potential to be an effective drug target in *T. brucei*. Here, we examined the effect of EGCG on *T. b. brucei* and found that physiologically relevant levels of EGCG had no effect on growth in culture and did not reduce virulence in a mouse model of infection, while higher levels of EGCG decreased TbACC activity, likely through an increase in TbACC phosphorylation. These data suggest that although EGCG inhibits *T. b. brucei* ACC activity, EGCG is a questionable candidate for further development as a potential cure for *T. brucei* infection. However, EGCG may be a useful pharmacological tool to investigate the signaling pathways governing phospho-regulation of TbACC.

RESULTS

To investigate whether EGCG could be used to target ACC in *T. b. brucei*, we first tested EGCG for its ability to inhibit TbACC enzymatic activity in whole cell lysates from two different life cycle stages: the procyclic form (PF) found in the fly midgut, and the mammalian bloodstream form (BF). Because of the known mode of action of EGCG on mammalian ACC phosphorylation, we sought to preserve any potential changes in phosphorylation caused by EGCG by adding HALT, a broad-spectrum phosphatase inhibitor, to the cell lysate. In the presence of HALT, EGCG reduced ACC activity in both BF and PF lysates, with an EC₅₀ of $29 \pm 1.3 \mu\text{M}$ (n=3) and $55 \pm 2.4 \mu\text{M}$ (n=3), respectively (Fig. 1A and B). HALT treatment alone had no effect on ACC activity (data not shown). However, in the absence of HALT, we observed no inhibition of TbACC activity in lysates by EGCG. Thus, EGCG treatment inhibited ACC activity only in the presence of phosphatase inhibitors. Although the effect of EGCG on ACC activity in lysates has not been previously measured, our observations are consistent with the reported mode of action of EGCG on ACC by increasing ACC phosphorylation (Collins *et al.*, 2007; Huang *et al.*, 2007; Moon *et al.*, 2007, Huang *et al.*, 2009; Murase *et al.*, 2009). That no effect was observed in the absence of HALT rules out a more general inhibitory “tannin” effect on the ACC enzyme (Wink 2008). Thus, we propose that HALT, by inhibiting the endogenous phosphatases in the lysate, preserved an EGCG-driven increase in ACC phosphorylation, resulting in decreased TbACC activity.

To more directly examine the effect of EGCG treatment on TbACC phosphorylation, we used BF and PF cell lines in which the C-terminus of one TbACC allele was fused to the c-myc epitope (TbACC-myc), which allows for expression of TbACC-myc at endogenous levels under the native promoter (Vigueira and Paul, 2011; and this study). BF and PF TbACC-myc lysates were treated with 100 μ M EGCG or DMSO as solvent control in the presence of HALT. After EGCG treatment, TbACC-myc immunoprecipitates were resolved by SDS-PAGE and phosphorylated TbACC detected by in-gel phospho-staining with Pro Q Diamond stain (Fig. 2A and B, upper panels, respectively). As a loading control, identically-loaded gels were transferred to nitrocellulose and total ACC detected by blotting with streptavidin-HRP (Fig. 2A and B, lower panels). Densitometry of the phospho-stained gels revealed that EGCG treatment resulted in a statistically significant 4.7 ± 0.12 -fold ($P < 0.0001$; $n = 3$) and 1.7 ± 0.20 -fold ($P < 0.05$; $n = 3$) increase in phosphorylation of TbACC in BFs and PFs, respectively. This increase in phosphorylation is correlated with the observed inhibition of TbACC activity in BF and PF lysates (Fig. 1A and 1B). It is possible that ACC-myc is inefficiently or aberrantly phosphorylated compared to native ACC, which might mask the true response of ACC to EGCG treatment. However, the facts that immunoprecipitated ACC-myc was also highly active and ACC-myc exhibited the same subcellular fractionation as native ACC (Vigueira and Paul, 2011) argue against this idea. In addition, similar increases in ACC phosphorylation were observed in mammalian cells after treatment with similar

concentrations of EGCG (50-400 μM) (Huang *et al.*, 2007; Moon *et al.*, 2007; Huang *et al.*, 2009; Murase *et al.*, 2009). Taken together, the results shown in Fig. 1 and 2 indicate that ACC is phosphorylated in both BFs and PFs of *T. brucei*, and that EGCG treatment leads to increased TbACC phosphorylation and correspondingly decreased TbACC activity. It is unknown how EGCG treatment leads to phosphorylation and inactivation of TbACC. In mammalian cells, how EGCG activates AMPK is not well understood, but occurs via activation of one or more upstream kinases (e.g. liver kinase B1 (LKB1) and calmodulin-dependent protein kinase (Cask) in a process that may involve the production of reactive oxygen species (ROS) via oxidation of EGCG (Sang *et al.*, 2005; Hou *et al.*, 2005; Hwang *et al.*, 2007; Collins *et al.*, 2007; Moon *et al.*, 2007; Murase *et al.*, 2009; Hou). Future work will be needed to determine the identity of the upstream TbACC kinase(s) and the role of ROS in their signaling.

We next examined the effect of EGCG on *T. b. brucei* growth in culture. Orally administered EGCG is rapidly cleared from the body (Ullmann *et al.*, 2003; Chow *et al.*, 2005). In humans, repeated daily oral administration of EGCG can achieve a maximum plasma concentration of $\sim 1 \mu\text{M}$ for 5-6 h (Ullmann *et al.*, 2004). A 6 day treatment of BF cells at these physiological EGCG concentrations (0.1–1 μM) caused no change in growth or doubling time (Fig. 3A and B). Next, we tested higher EGCG concentrations (5–50 μM), and observed a statistically significant reduction in cell growth over 48 h (Fig. 3C and D), with an EC_{50} of $34 \pm 2.4 \mu\text{M}$ and $25 \pm 2.7 \mu\text{M}$ for BF and PF parasites, respectively ($P < 0.01$,

n=4). These values are consistent with the trypanocidal activity of EGCG (IC₅₀ 20.2 μM at 72 h) previously reported for *T. b. rhodesiense* (Tasdemir *et al.*, 2006). Our previous data showed that TbACC is largely unnecessary in BF parasites cultured *in vitro* and is only required when PF parasites are cultured in low-lipid media (Vigueira and Paul, 2011). Thus, we contend that inhibition of TbACC by EGCG should have little consequence on *T. b. brucei* growth in culture. Consequently, we attribute the reduction in growth observed with EGCG concentrations >5 μM to other previously described target(s) of EGCG (Ahmed, 2010; Singh *et al.*, 2011), rather than to its inhibition of TbACC.

Finally, we examined the effect of EGCG treatment on the course of *T. b. brucei* infection in mice. Although the EC₅₀ values for EGCG for growth in culture are greater than the achievable physiological concentrations in humans, there were three reasons to justify testing EGCG in a mouse model of infection. First, *T. b. brucei* exhibited condition-specific essentiality in the case of both TbACC and enoyl-CoA reductase (EnCAR). PF ACC and EnCAR RNAi cell lines exhibited slowed growth only when exogenous lipids were limited, and a TbACC RNAi line had attenuated virulence in mice (Lee *et al.*, 2006; Vigueira and Paul, 2011). Second, studies in rats showed that intravenous administration of EGCG resulted in greater plasma half-life and in higher maximal plasma concentrations than seen with the oral route of administration (Isbrucker *et al.*, 2006; Misaka *et al.*, 2012). Third, previous studies of EGCG in mice have yielded promising results for treatment of trypanosomiasis. *T. cruzi* mortality in mice and growth in

culture was reduced with EGCG treatment (Paveto *et al.*, 2004; Güida *et al.*, 2007). In addition, inflammation caused by *T. b. brucei* infection was reduced and survivorship was extended with oral green tea supplementation (Karori *et al.*, 2008). Taken together, these observations suggest that first, parenteral administration of EGCG might achieve therapeutic plasma concentration of EGCG, and second, the requirement for ACC and thus the parasite's sensitivity to EGCG could be higher *in vivo*.

To test the ability of EGCG to clear a *T. b. brucei* infection in mice, we administered daily IP injections of 4.13 mg/kg EGCG in sterile H₂O two days prior to infection and over the course of the trial. This EGCG concentration had been previously determined not to cause mouse liver damage, though the actual EGCG serum concentration was not determined (Güida *et al.*, 2007). After 2 days of EGCG pre-treatment, Swiss mice were then infected with 1x10⁵ BF wild-type 427 parasites as previously described (Bacchi *et al.*, 2009), and time until death (or humane end-point) was determined. In this infection model, EGCG treatment had no effect on infection duration or mouse mortality. Mean time until death was 3.5 days for both treatment and control groups (data not shown). Thus, although EGCG has trypanocidal activity at supraphysiological concentrations, the compound did not attenuate virulence in our acute infection mouse model. EGCG concentrations may not have been sufficiently high in the mouse bloodstream to affect the parasite, however a much lower intraperitoneal dose (0.8 mg/kg/day) led to a significant decrease in parasitemia and increased

survivorship of mice infected with *T. cruzi* (Güida *et al.*, 2007). Alternatively, it is possible that the course of infection was too rapid to allow EGCG to exert its effects. If so, a chronic infection model might be better suited to examine the efficacy of EGCG as a possible anti-trypanosomiasis therapy.

In summary, we found that EGCG inhibits TbACC activity in lysates, and this inhibition was correlated with an increase in ACC phosphorylation. This suggests that EGCG may be a useful tool for studying the effects of phosphorylation on TbACC activity. We also demonstrated that EGCG kills both PF and BF parasites in culture. However, EGCG treatment did not affect *T. b. brucei* virulence in a mouse model of acute infection, which reduces its promise as a therapeutic candidate to treat *T. brucei* infection.

METHODS

Materials, cell lines, and media

Most chemicals and reagents were purchased from Thermo Fisher Scientific and Sigma, including EGCG (E4143), which was prepared and frozen in single-use aliquots, either as a 100X stock in dimethyl sulfoxide (DMSO) or in sterile water for use in mice. EGCG stocks were freshly thawed just before use. Minimum Essential Medium Eagle and Iscove's Modified Dulbecco's Medium, was from Invitrogen. Serum Plus was from JRH Biosciences. [¹⁴C]NaHCO₃ (14.9 mCi mmol⁻¹) was from American Radiolabeled Chemicals. Wild-type (WT) *T. b. brucei* 427 bloodstream form (BF) and tsetse midgut procyclic form (PF) cell

lines were a kind gift of Dr. Paul Englund (Johns Hopkins School of Medicine). BFs were grown at 37°C/5% CO₂ in HMI-9 media containing 10% heat-inactivated fetal bovine serum (FBS) and 10% Serum Plus (Hirumi and Hirumi, 1989). PFs were grown at 28°C/5% CO₂ in SDM-79 media containing 10% heat-inactivated FBS and 7.5 µg ml⁻¹ hemin (Brun and Shonenberger, 1979). PF ACC-myc cells are tagged in one genomic locus with a c-terminal fusion to the c-myc epitope and have been described previously (Vigueira and Paul, 2011). BF ACC-myc cells were generated in this study with the same tagging construct used to make the PF ACC-myc cell line. ACC-myc cell lines are maintained in the appropriate growth media supplemented with 2.5 µg ml⁻¹ phleomycin.

Effect of EGCG on *T. b. brucei* growth

WT BF *T. b. brucei* were diluted into fresh media containing 0.1-1 µM EGCG or 1% DMSO as the solvent control and the cell culture densities measured every 2 days for 6 days by flow cytometry (BD FACScan). Following each cell count, cultures were diluted to maintain logarithmic phase growth, and EGCG/DMSO was added to maintain experimental concentrations. Doubling times were calculated from the growth curves.

To determine EC₅₀ for growth, WT BF and PF *T. brucei* were diluted into fresh media containing 5-50 µM EGCG or 1% DMSO as solvent control and cell culture densities determined after 2 days.

ACC activity assays

Hypotonic lysates were desalted and TbACC activity was assayed essentially as described (Vigueira and Paul, 2011). Briefly, ACC activity was determined by measuring the incorporation of the [¹⁴C]CO₂ from [¹⁴C]NaHCO₃ into the acid resistant [¹⁴C]malonyl-CoA product, which is quantified by scintillation counting. To test the effect of EGCG on TbACC activity, 5-100 μM EGCG or 1% DMSO only (solvent control) was added to the lysates with or without 1X HALT phosphatase inhibitor cocktail (Thermo Scientific Pierce) just prior to assaying for TbACC activity.

ACC phosphorylation and blotting

Hypotonic lysates from BF and PF ACC-myc cells were prepared in the presence of 1X HALT phosphatase inhibitor cocktail. Lysates were supplemented with 5 mM ATP, divided, with one aliquot treated with 100 μM EGCG and the other with 1% DMSO as solvent control. Lysates were then incubated at 30°C for 30 min. under constant mixing (Eppendorf Thermomixer, 500 rpm). ACC-myc was immunoprecipitated from the lysates using the ProFound c-myc IP/Co-IP kit (Thermo Scientific Pierce) according to the manufacturer's instructions and eluted in 150 mM glycine at pH 2.2 followed by immediate neutralization with 9.5 M Tris-HCl, pH 9.0.

To detect phosphorylated ACC-myc, approximately equal quantities of immunoprecipitated ACC-myc (~8.8 x 10⁷ cell equivalents/lane for PF and ~1.5 x

10^8 cell equivalents/lane for BF) were resolved by SDS-10%PAGE, followed by gel staining with Pro Diamond Q phosphoprotein gel stain (Invitrogen) according to the manufacturer's instructions. Phospho-stained gels were imaged using the Gel Doc XR Imaging System (BioRad). To detect total ACC, identically loaded non-stained gels were transferred to nitrocellulose and probed for ACC as described (Vigueira and Paul, 2011) with streptavidin conjugated to horseradish peroxidase (SA-HRP), which detects the ACC biotin prosthetic. Densitometric quantification was performed on gels and appropriately exposed blots (in the linear range of detection) using Image J software (NIH). Phosphorylated ACC-myc was normalized to the total amount of ACC in the ACC-myc immunoprecipitates and lysates.

Mouse infection

To test the ability of EGCG to clear a *T. b. brucei* infection in mice, we chose a daily dose of 4.13 mg/kg EGCG prepared in sterile H₂O and administered daily by IP injection. This EGCG concentration had been previously determined not to cause mouse liver damage, though actual serum levels of EGCG were not determined (Güida *et al.*, 2007). For the EGCG-treated group, 12 week old Swiss mice were pre-treated with EGCG for two days prior to infection and EGCG treatment was continued throughout the course of infection. Both the EGCG-treated (n=5) and untreated controls (n=5) were infected with 1×10^5 BF wild-type 427 parasites as previously described (Bacchi *et al.*, 2009).

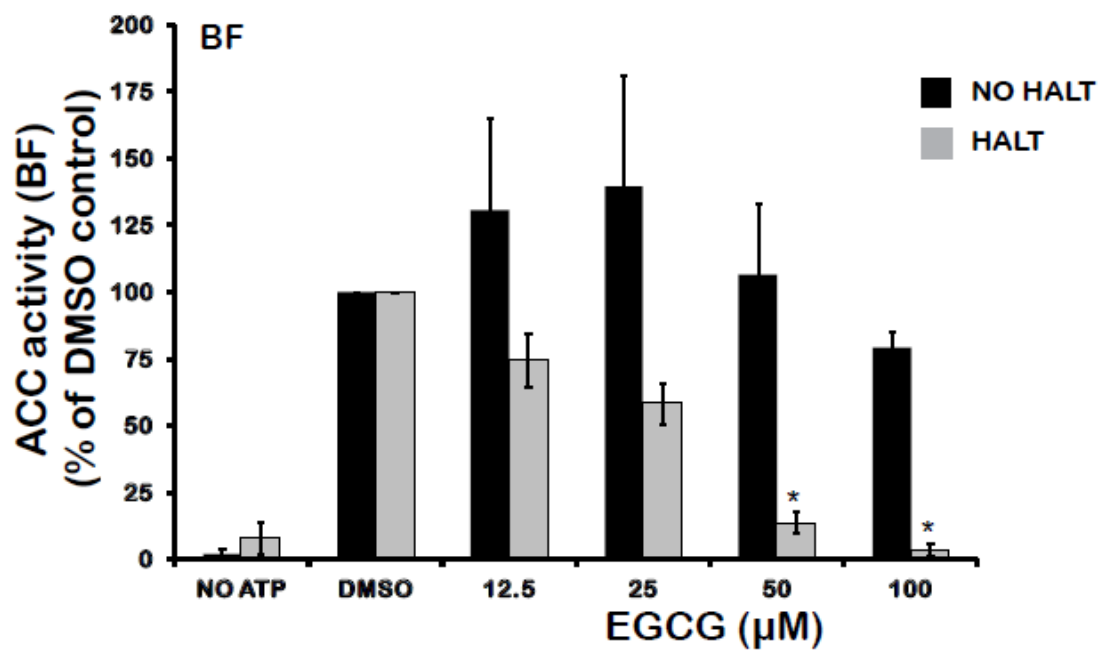
Tail blood samples were examined by microscopy to confirm successful infection, and time to death (or humane end-point) was determined. All work with animals was carried out in compliance with a protocol approved by the Clemson University Institutional Animal Care and Use Committee.

FIGURES

Figure 3.1 Inhibition of TbACC activity by EGCG. TbACC activity in lysates

of (A) BF and (B) PF trypanosomes was measured in the presence of 5–100 μM EGCG in the absence (black bars) or presence (grey bars) of HALT phosphatase inhibitor cocktail. As a negative control, ATP was omitted from the reaction (No ATP). Values are expressed as a percentage of the DMSO solvent control. DMSO concentrations were maintained at 1% v/v for all conditions. The mean of 3 independent experiments is shown. Error bars indicate $\pm\text{SEM}$. The * indicates $P < 0.01$ for the difference between DMSO control and EGCG-treated conditions (student's *t*-test).

A



B

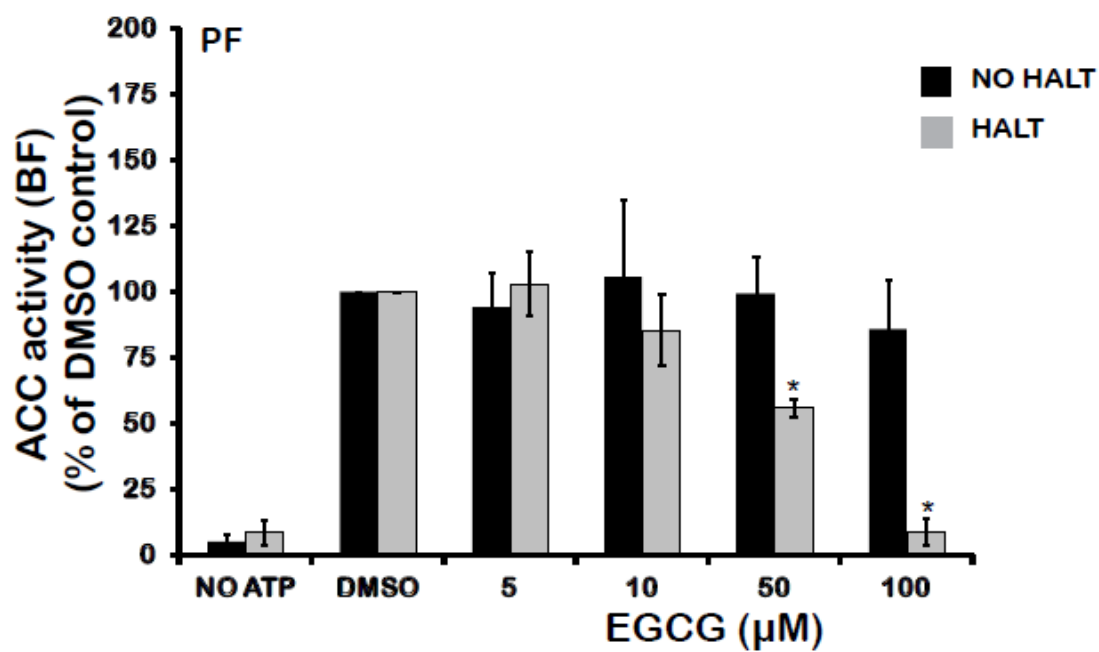


Fig 3.2 EGCG induces phosphorylation of TbACC. BF and PF ACC-myc lysates were treated with 100 μ M EGCG or DMSO as solvent control in the presence of HALT phosphatase inhibitor cocktail. (A) BF and (B) PF TbACC-myc immunoprecipitates ($\sim 5 \times 10^8$ cell equivalents/lane) were resolved by SDS-PAGE and stained with Pro Q Diamond phosphoprotein gel stain to detect phosphorylated ACC (ACC-p; upper panels). Identically loaded gels were blotted to nitrocellulose and probed for total ACC with SA-HRP (ACC-t; bottom panels). Representative gels and blots are shown. Unlabeled upper band in panel A is an unknown phosphorylated contaminant. (C) Densitometric quantification of phosphorylated ACC from phospho-stained gels normalized to total ACC detected by SA-HRP blotting. The mean of 3 independent experiments is shown. Error bars indicate the \pm SEM. The * indicates $P < 0.05$ and ** indicates $P < 0.00001$ for the difference between DMSO control and EGCG-treated conditions (student's *t*-test).

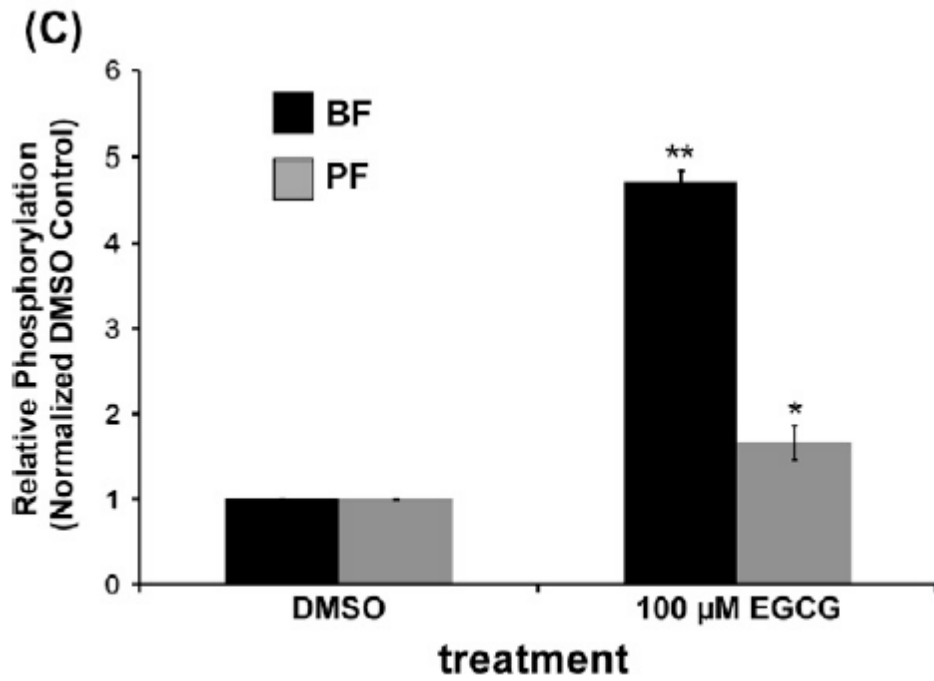
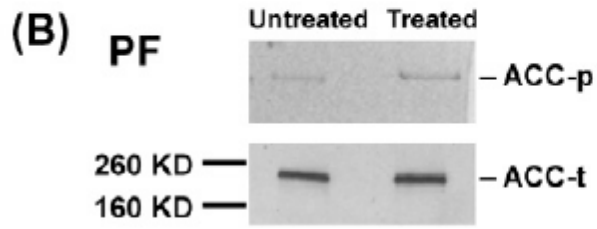
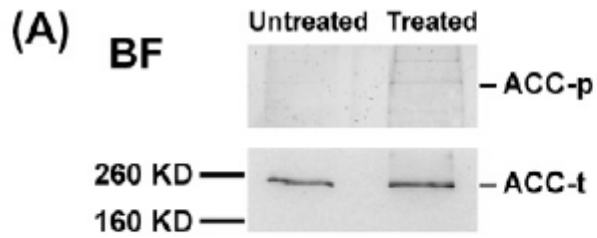
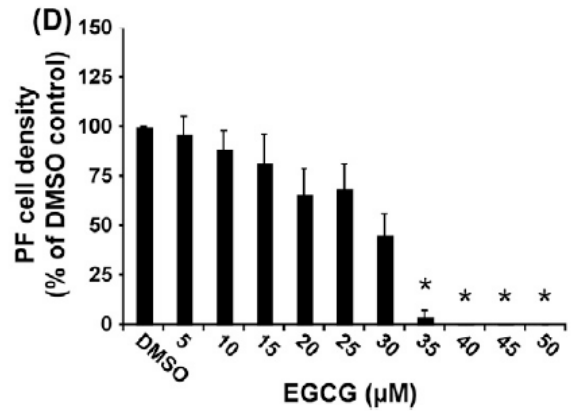
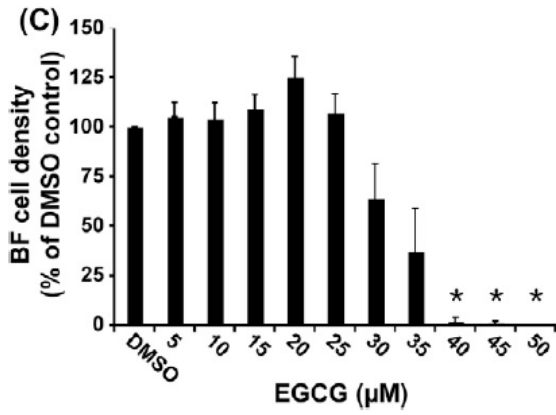
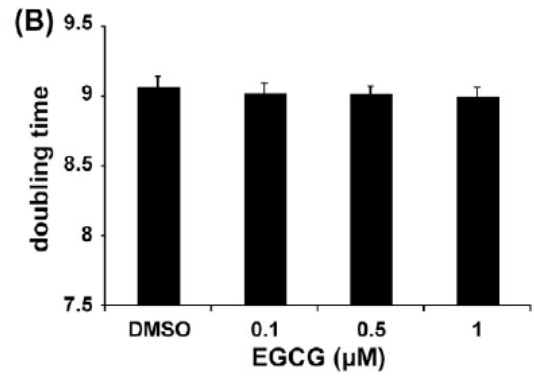
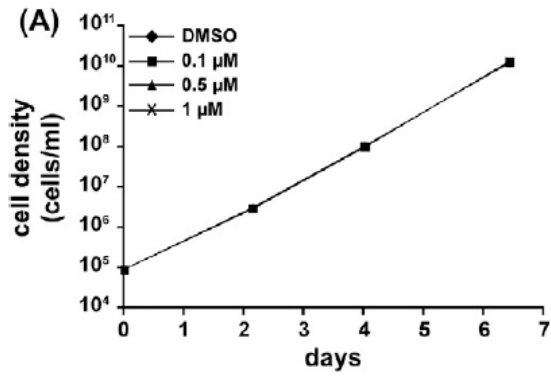


Fig 3.3 Effect of EGCG on in vitro growth of *T. brucei*. (A and B) WT BF cells were diluted back to 1×10^6 cells/ml into fresh media containing 0.1–1 μ M EGCG or DMSO as the solvent control and culture cell densities were monitored for 6 days. (A) Representative growth curve showing cumulative culture densities. (B) Mean culture doubling times of 3 independent experiments. Error bars show \pm SEM. (C) WT BF and (D) WT PF cells were back-diluted into fresh media containing 5–50 μ M EGCG or DMSO control. Culture cell densities were determined after 48 h. Values are expressed as a percentage of DMSO control. The mean of 4 independent experiments is shown. Error bars show \pm SEM. The * indicates $P < 0.01$ for the difference between DMSO and EGCG-treated conditions (student's *t*-test).



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CHAPTER 4

ALLOSTERIC REGULATION OF *Trypanosoma brucei* ACETYL-COA

CARBOXYLASE

INTRODUCTION

Trypanosoma brucei is a protozoan extracellular parasite that is introduced into the bloodstream of a mammalian host by its tsetse fly vector during a blood meal. From the mammalian bloodstream, the parasite crosses the blood brain barrier and passes into the central nervous system. *T. brucei* causes a fatal disease in humans known as African sleeping sickness and a wasting disease in livestock known as Nagana. If not treated, this disease is nearly 100% fatal. Currently available drugs are highly toxic and resistance is a growing problem. Hence, there is a need to find novel therapeutics (Jacobs *et al.*, 2011).

Throughout its life cycle, the parasite experiences different nutrient levels in the microenvironments of its hosts. In these microenvironments, the parasite undergoes physiological changes in order to adapt to its host. For example, *T. brucei* bloodstream forms (BFs) upregulate genes such as pyruvate kinase 1 and hexokinase 1, while tsetse midgut procyclic forms (PFs) upregulate phosphoglycerate kinase and the cytochromes (Jenson *et al.*, 2009).

Both BF and PF *T. brucei* require fatty acids (FAs) and can readily take FAs from their hosts (Coppens *et al.*, 1995; Green *et al.*, 2003). Under conditions of limited environmental FAs, however *T. brucei* can also synthesize

FAs *de novo* (Dixon *et al.*, 1971; Morita *et al.*, 2000). The first committed step in FA synthesis is catalyzed by acetyl-CoA carboxylase (ACC), which converts acetyl-CoA to malonyl-CoA, the two carbon donor for the ER-localized FA synthesis pathway (Lee *et al.*, 2006). However, FA synthesis is an energy intensive biosynthetic pathway. For example, the parasite would need to expend 6 ATP and 12 NADH/NADPH reducing equivalents to synthesize a 16-carbon FA, while uptake and activation of the same FA from the host might require only 1 ATP. As FA synthesis is such an energy-utilizing process, it likely to be important for *T. brucei* to regulate the balance between FA synthesis and uptake.

Because it catalyzes the first committed step in FA synthesis, ACC plays a central role in lipid metabolism in other organisms and has been shown to be controlled by multiple regulatory mechanisms (Lane *et al.*, 1974; Moule *et al.*, 1992; Anderson *et al.*, 1998; Andre *et al.*, 2012). Most ACCs are subject to allosteric regulation by metabolites upstream (feed-forward signals) and regulation by downstream products (feedback signals) (Andre *et al.*, 2012). ACC is also known to undergo conformational changes that modulate its activity (Vagelos *et al.*, 1963; Lane *et al.*, 1974). Metabolites such as citrate, glutamate, and other dicarboxylic acids activate ACC (Vagelos *et al.*, 1963; Lane *et al.*, 1974), while fatty acyl-CoAs function as negative regulators (Moule *et al.*, 1992; Anderson *et al.*, 1998).

In this chapter, I examined allosteric regulation of TbACC and the effect of different modulators on TbACC activity: adenine nucleotides such as cAMP and

AMP; metabolic intermediates such as pyruvate, citrate, glutamate, and acetate; and fatty acyl-CoAs. I also assessed the oligomeric state of TbACC under normal lipid growth conditions. I found that TbACC activity was reduced when treated with increasing concentration of AMPK. TbACC showed increased activity in the presence of metabolic intermediates, with highest activity when treated with citrate and moderate increase when treated with acetate and glutamate. TbACC was inhibited by fatty acyl-CoA in a chain-length dependent manner. I also demonstrated that TbACC potentially, might form oligomers.

RESULTS

AMP decreased and cAMP increased TbACC activity

AMP-activated protein kinase (AMPK) and protein kinase A (PKA) were both previously known to affect ACC in other organisms (Boone *et al.*, 1999; Park *et al.*, 2002), and homologs to both kinases were present in *T. brucei* (Clemmens *et al.*, 2009; Kramer, 2005). As AMPK and PKA were responsive to AMP and cAMP, respectively, we first examined the effect of these adenine nucleotides on TbACC activity. PF WT lysates were assayed for ACC activity as described previously (Vigueira and Paul, 2011) in the presence of a 12.5–100 μ M AMP and cAMP (see Table 4.1). TbACC showed a decrease in activity with increasing concentrations of AMP (~40% reduction at the highest concentration tested (100 μ M)) (Fig. 4.1). In contrast, cAMP treatment increased TbACC activity, and this

increase was only significant at the highest concentration of cAMP tested (100 μM) (Fig 4.1).

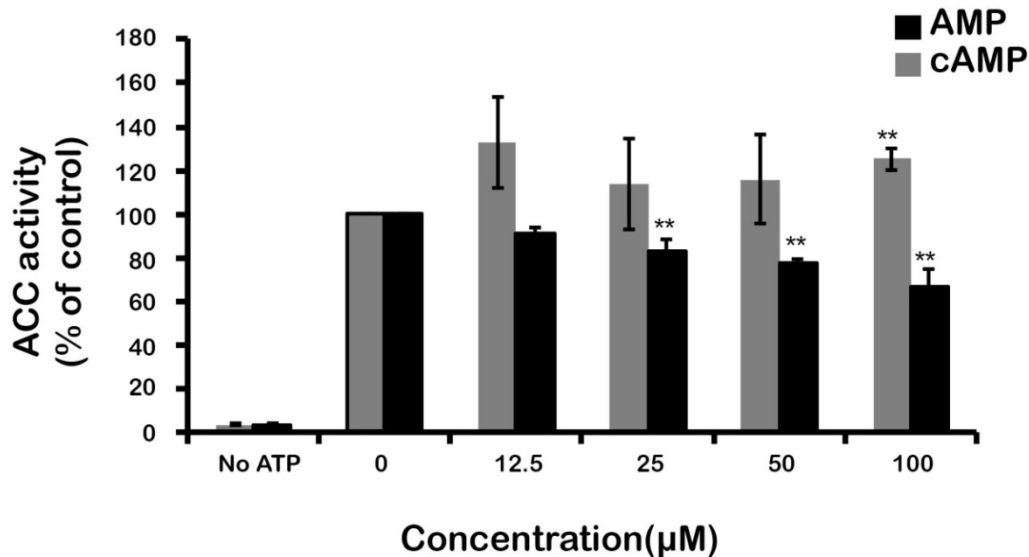


Figure 4.1: AMP and cAMP affected PF TbACC activity. Hypotonic lysates prepared from WT PFs were treated with 12.5–100 μM AMP (black bars) and cAMP (grey bars) and assayed for TbACC activity. As a negative control, ATP was omitted from the reaction (No ATP). Values are expressed as a percentage of the untreated control. The mean \pm SEM of 3 independent experiments is shown. ** $P < 0.01$ for the difference between control and treatment (Student's t -Test).

Citrate increased TbACC activity

Next we examined the effect of intermediate metabolites on TbACC activity. WT PF lysates were treated with citrate, pyruvate, glutamate, or acetate, and assayed for TbACC activity. Citrate caused a dose-dependent increase in TbACC activity (Fig. 4.2A), while glutamate and acetate had a small but

significant positive effect on TbACC activity (Fig. 4.2B). Pyruvate treatment had no apparent effect on TbACC activity except at 10 mM (Fig. 4.2A).

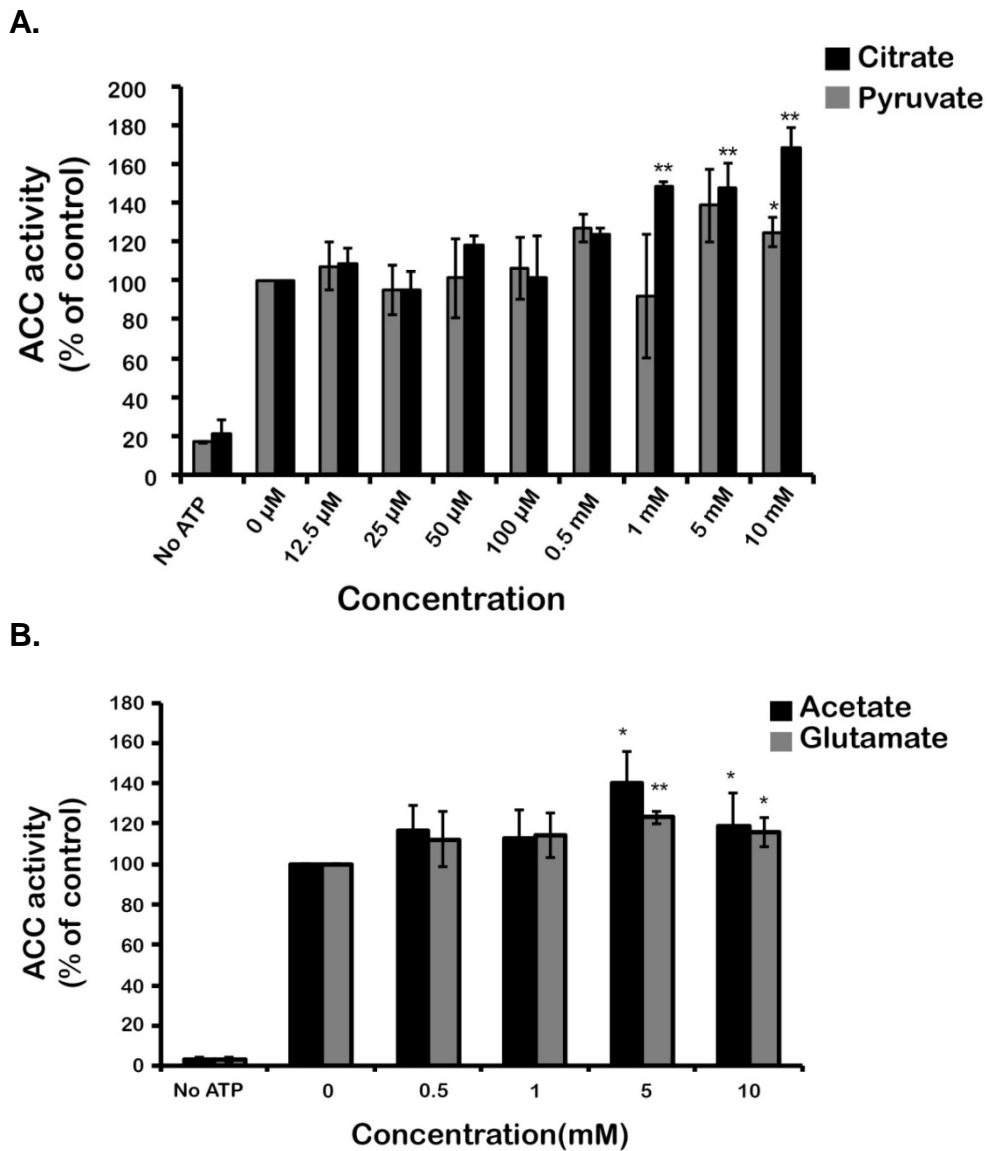


Figure 4.2: Citrate increased TbACC activity. WT PF hypotonic lysates were treated with **(A.)** 12.5 μ M–10 mM citrate (black bars) and pyruvate (grey bars) or **(B.)** 0.5–10 mM acetate (black

bars) and glutamate (grey bars) and assayed for TbACC activity. As a negative control, ATP was omitted from the reaction (No ATP). Values are expressed as a percentage of untreated control. The mean \pm SEM of 3 independent experiments is shown. * $P < 0.05$ and ** $P < 0.01$ for difference between control and treated conditions (Student's t -test).

Fatty acyl-CoAs inhibited TbACC activity in a chain-length dependent manner Mammalian ACCs are subject to feedback inhibition by the product of FA synthesis, palmitoyl-CoA. Thus, we examined whether TbACC was also inhibited by fatty acyl-CoAs and if there was a dependence on acyl-chain length. WT PF lysates were treated with fatty acyl-CoAs 12–18 carbons in length and assayed for TbACC activity. TbACC was inhibited by fatty acyl-CoAs, with the strongest inhibition observed with C16 palmitoyl-CoA and C18 stearoyl-CoA (EC_{50} s of 12.6 μ M and 12.5 μ M, respectively) (Fig. 4.3). TbACC was less sensitive to C14 myristoyl-CoA (EC_{50} =40.6 μ M) and largely refractory to C12 lauroyl-CoA.

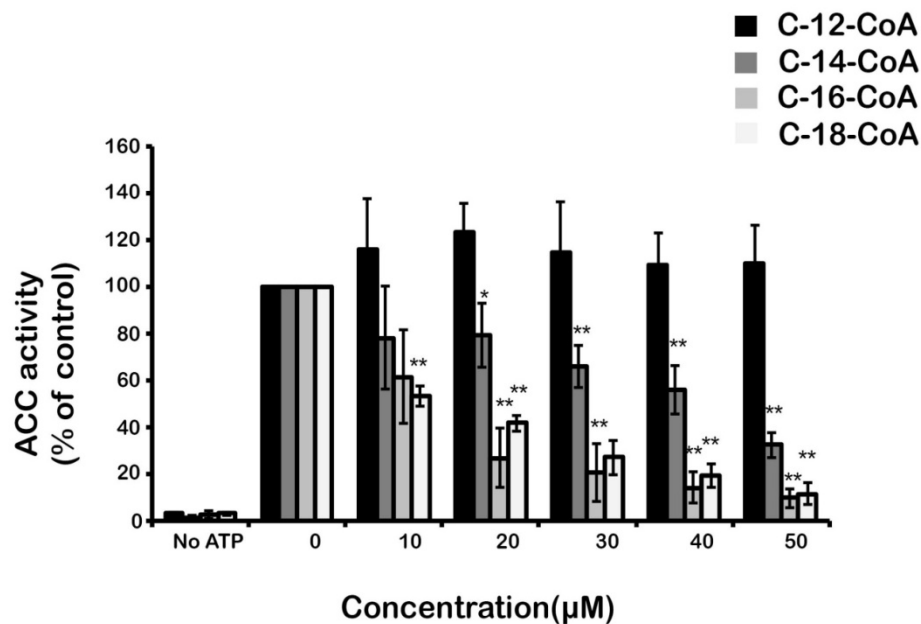


Figure 4.3: Fatty acyl-CoAs inhibit PF TbACC activity in lysates. WT PF hypotonic lysates were treated with 10–50 µM fatty acyl-CoAs and assayed for TbACC activity. As a negative control, ATP was omitted from the reaction (No ATP). Values are expressed as a percentage of the untreated control. The mean ± SEM of 3 independent experiments is shown. * $P < 0.05$ and ** $P < 0.01$ for the difference between control and treated conditions (Student's *t*-Test).

TbACC exists in oligomers

Mammalian and avian ACCs form polymers which suggests that TbACC also might form higher order structures. Thus, we determined the oligomeric structure of TbACC using size exclusion chromatography. Hypotonic lysates prepared from WT PFs grown in normal lipid media were fractionated on an S300 sephacryl column equilibrated in BC buffer. Column fractions were blotted for total ACC using SA-HRP (Fig. 4.4C). TbACC was found in complexes estimated to range between 400-1000 kDa when compared to a standard curve generated

from molecular weight standards sized on the same column under the same buffer conditions (Fig. 4.4A and B). Given a predicted molecular weight of 243 kD for TbACC, the gel filtration data suggests that TbACC might exist as a mixture of oligomers, which correspond in size to dimers, trimers, and tetramers of TbACC.

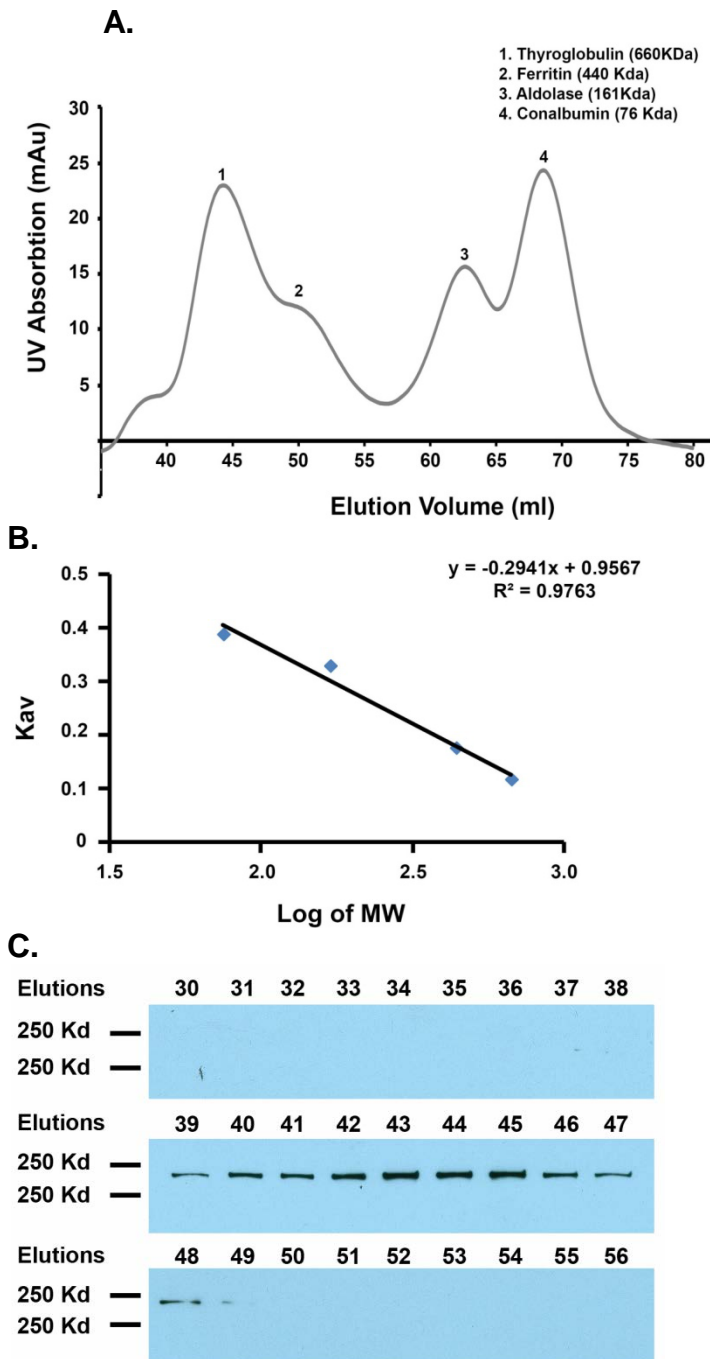


Fig 4.4: TbACC exists in oligomers. PF WT hypotonic lysates were resolved by Sephacryl S-300 size exclusion chromatography and TbACC detected in column fractions by SA-HRP blotting. **(A.)** Abs280 nm of indicated protein standards **(B.)** Standard curve of the log molecular weights vs. distribution coefficient (K_{av}). **(C.)** SA-HRP blotting of column fractions for total TbACC.

DISCUSSION

We hypothesized that TbACC would be subject to allosteric regulation, as this is true of ACC in all other organisms studied to date (Vagelos *et al.*, 1963; Lane *et al.*, 1974). We examined the effect of three classes of potential allosteric regulators on TbACC activity in WT PF hypotonic lysates. We first examined the effect of adenine nucleotides (AMP and cAMP) for their effect on TbACC activity. TbACC activity was only mildly stimulated by cAMP. In contrast, TbACC was significantly inhibited by AMP in a dose-dependent manner. It is unclear whether this inhibition of TbACC by AMP is due to a direct or indirect effect on TbACC. AMP could be competing for ATP-binding in the active site, though the assay conditions have a 10-fold higher concentration of ATP (0.5 mM). AMP could also be binding to a second regulatory site of TbACC. Finally, AMP could be acting indirectly through the activation of AMPK, which is known to phosphorylate and inhibit ACC in other organisms. This mode of action is supported by our studies in Ch. 3 that showed the AMPK activator EGCG inhibited PF TbACC with increasing concentration in a phosphorylation-dependent manner.

As metabolic intermediates reflect the nutritional state of an organism, we also examined the effect of intermediate metabolites (acetate, citrate, glutamate and pyruvate) on TbACC activity. We found that pyruvate did have a stimulatory effect on TbACC activity, though only at the highest concentration tested (10 mM). To our knowledge, this is the first reported stimulation of ACC by pyruvate. In most cells, including *T. brucei*, pyruvate is the major metabolic source of

acetyl-CoA for FA synthesis. Pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase complex (PDH) followed by the transport of acetate back to the cytosol via the citrate/malate shuttle (Hatzivassiliou *et al.*, 2005). There is some evidence for this pathway operating in *T. brucei* (Riviere *et al.*, 2009). These experiments elucidated an alternate pathway in *T. brucei* involving the conversion of pyruvate to acetyl-CoA within the mitochondrion by PDH and subsequent conversion to acetate by an acetate:succinate-CoA transferase, followed by transport of acetate into the cytosol by an unknown transporter where an acetyl-CoA synthetase rapidly re-esterifies it to acetyl-CoA (Rivière *et al.*, 2009). Thus, pyruvate may serve as feed-forward signal reflecting high nutrient status in the parasite and the need to synthesize FAs and the precursors needed to synthesize lipids (Fig. 4.5).

We found that citrate had the most significant effect of the metabolites tested, stimulating TbACC activity in a nearly dose-dependent manner. The existence of a citrate-dependent alternate pathway for acetyl-CoA production from citrate is already known in *T. brucei*, which might explain why citrate had a significant effect on TbACC activity (Rivière *et al.*, 2009) (Fig. 4.5). This citrate stimulation of TbACC is also in accordance with mammalian and avian ACCs, which are known to be activated by citrate and glutamate (Beatty and Lane *et al.*, 1983). In addition, citrate and glutamate were shown to induce polymerization of ACC thereby increasing its activity (Beatty and Lane *et al.*,

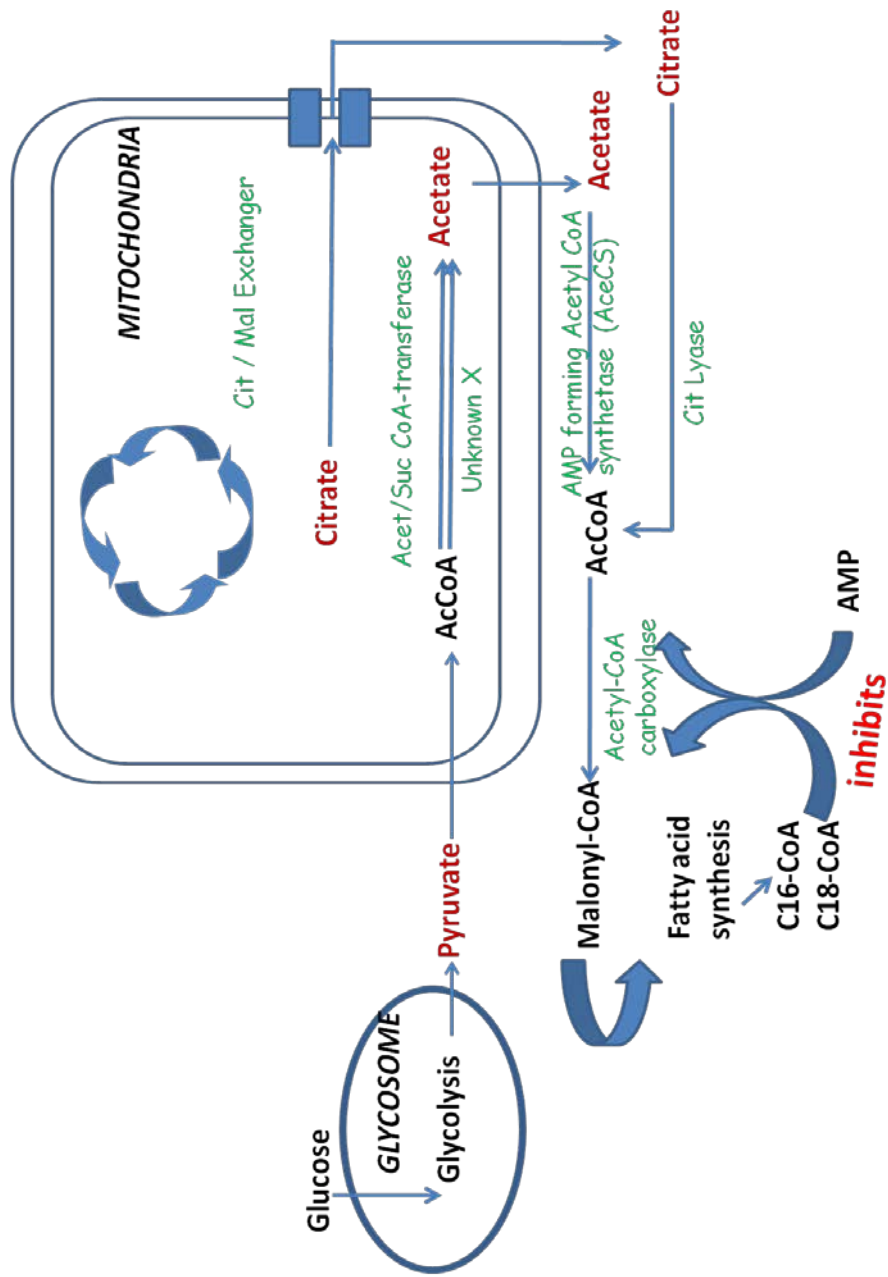


Fig 4.5: *T. brucei* metabolic pathways showing different allosteric regulators and their effect on TbACC

1983; Cesquini *et al.*, 2008). TbACC was only mildly stimulated by glutamate, suggesting it may not be as sensitive to feed-forward signals of amino acid metabolism. A key enzyme in glutamate metabolism is glutamate dehydrogenase (Newsholme *et al.*, 2003). However, earlier studies in *T. brucei* showed that knockout of glutamate dehydrogenase had no effect on the parasite (Estevez *et al.*, 1999). Perhaps other amino acids, such as proline or threonine, might be more relevant to TbACC regulation in *T. brucei*, as proline metabolism is already known to be important in PFs (Coustou *et al.*, 2008; Bringaud *et al.*, 2012) and threonine can serve as a source of acetyl-CoA for FA synthesis (Linstead *et al.*, 1974). Finally, acetate also showed a mildly stimulating effect on TbACC, which might be due to activation of AMP-forming acetyl-CoA synthetase (Fig. 4.5). However, the concomitant generation of AMP, which we have already shown inhibits ACC, may explain the mild effect of acetate.

Fatty acyl-CoAs are the final products of FA synthesis and thus, we examined their effect on TbACC activity. TbACC exhibited an acyl chain-length dependent inhibition of activity, with longer chain lengths such as palmitoyl-CoA (C16) and stearoyl-CoA (C18) showing more robust inhibition, followed by myristoyl-CoA (C14). Lauroyl-CoA (C12) had no effect on TbACC activity. This chain-length specificity of fatty acyl-CoA action might be explained by the FA requirements of PFs. PFs express procyclin as their surface coat protein and are attached to the plasma membrane by a GPI-anchor that contains palmitate (C16) and stearate (C18) FAs (Butikofer *et al.*, 1997). Hence, in PFs there is a demand

for longer chain FAs and thus FA synthesis is not affected by the shorter FAs. Mammalian hepatic ACC is known to bind reversibly to palmitoyl-CoA resulting in inhibition of the enzyme (Ogiwara *et al.*, 2008). To our knowledge the chain-length specificity of this inhibition in mammalian ACCs has not been explored.

Mammalian and avian ACCs polymerize in response to both allosteric regulators and dephosphorylation (Vagelos *et al.*, 1963; Lane *et al.*, 1974). In yeast, an ACC mutant was shown to form planar arrays (Schneiter *et al.*, 1996), suggesting ACC may also polymerize in yeast. Finally, immunofluorescence microscopy studies of TbACC demonstrated that the enzyme is localized to numerous cytoplasmic puncta (Vigueira and Paul, 2011). Based on these observations, we examined the oligomeric state of TbACC by size exclusion chromatography. In PFs, TbACC was found to exist in complexes that migrated with a molecular weight range of 400-900 KDa, consistent with a mixture of dimers, trimers, and tetramers. Detection of TbACC oligomers suggested the possibility that TbACC might be regulated at the level of quaternary structure in addition to regulation by phosphorylation and at the level of protein expression. However, the fractionation was performed with lysate rather than purified protein, thus we cannot rule out the role of other proteins interacting with TbACC (Magnard *et al.*, 2002). We also cannot rule out the possibility that TbACC formed aggregates, but sub-cellular fractionation of TbACC performed under similar conditions showed that TbACC behaved as a soluble cytosolic protein and no indication of aggregation was observed (Vigueira *et al.*, 2011). The

TbACC oligomers isolated in our studies were too small to consist of the puncta seen by microscopy. A possible explanation for this disparity may be that the TbACC oligomers are unstable in lysates and hence, the higher-order polymers break into the smaller protomers we observed by gel filtration. In mammals, ACC binds to a 22 kDa cytosolic protein, MIG12, which stabilizes the ACC polymer (Kim *et al.*, 2010). No MIG12 homolog was found in the *T. brucei* genome, which might also explain the relative instability of the larger TbACC structures.

In summary, I have shown that TbACC activity is inhibited by AMP and long-chain fatty acyl-CoAs and stimulated by citrate and to a lesser extent, acetate and glutamate indicating a degree of metabolic control of TbACC in response to the nutritional state of the cell. The extent to which this regulation involves direct binding to TbACC remains to be determined. Finally, TbACC might exist as a mixture of oligomers in PFs grown in normal media. However this will need to be confirmed with purified protein and sedimentation studies. If TbACC oligomers are confirmed, it will be interesting to determine if the TbACC oligomeric state is affected by growth in high or lipid media or in response to the effectors such as AMP and glutamate, which we already demonstrated alter TbACC activity in lysates.

METHODS

Effect of allosteric regulators on TbACC activity

WT PFs were grown to mid-logarithmic stage. Hypotonic lysates were prepared from 5×10^8 cells and chromatographed through a Sephadex G50 column to remove endogenous substrates. The resulting lysates were treated with different concentrations of allosteric regulators (Table 4.1) and TbACC activity assayed essentially as described (Vigueira and Paul, 2011), except the final acetyl-CoA concentration was 2.5 mM. Values were normalized to the “No ATP” control and expressed as a percentage of untreated lysate. Significance of differences between treatment and control were determined using the Student's t- Test.

Table 4.1 Tested concentrations of potential allosteric regulators

Class	Name	Concentrations
Adenine nucleotides		
	cAMP	0, 12.5, 25, 50, 100 μ M
	AMP	0, 12.5, 25, 50, 100 μ M
Metabolic intermediates		
	Pyruvate	0, 12.5, 25, 50, 100 μ M 0.5, 1, 5, 10 mM
	Citrate	0, 12.5, 25, 50, 100 μ M 0.5, 1, 5, 10 mM
	Acetate	0, 0.5, 1, 5, 10 mM
	Glutamate	0, 0.5, 1, 5, 10 mM
Fatty acyl-CoAs		
	Lauroyl-CoA	0, 10, 20, 30, 40, 50 μ M
	Myristoyl-CoA	0, 10, 20, 30, 40, 50 μ M
	Palmitoyl-CoA	0, 10, 20, 30, 40, 50 μ M
	Stearoyl-CoA	0, 10, 20, 30, 40, 50 μ M

Sizing of TbACC oligomers

PF WT hypotonic lysates were prepared from cells grown in normal lipid conditions. Size exclusion chromatography was performed using a Sephacryl S-300 column and the AKTA Prime Plus chromatography and purification system (GE Healthcare) for sample injection, separation, and fraction collection. The column was equilibrated in BC Buffer (50 mM Tris-Cl pH 8.0, 5 mM $MgCl_2$, 5 mM DTT) and run in the same buffer. Samples were injected in a volume of 800 μ l,

separated at a flow rate of 0.5 ml min^{-1} , and 1 ml fractions were collected at room temperature. The column fractions were resolved by SDS-PAGE and total ACC detected by SA-HRP blotting. Calibration of the column was performed using a standard curve generated by the separation of blue dextran (void volume) and the following protein standards: Thyroglobulin (660 KDa), Ferritin (440 KDa), Aldolase (161 KDa) and Conalbumin (76 KDa). Protein peaks were monitored using the UV detector at 280 nm.

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CHAPTER 5

CONCLUDING REMARKS

Trypanosoma brucei is a protozoan extracellular parasite that is transmitted by infected tsetse flies. *T. brucei* affects sub-Saharan Africa, where it causes human African trypanosomiasis (HAT). *T. brucei* also causes a wasting disease in livestock called Nagana, which makes agriculture very difficult and has caused this region of Africa to be nicknamed the “green desert”, owing to the tremendous economic impact *T. brucei* has had on the people living in this poverty stricken continent (FAO, 2002). The survival of *T. brucei* within its hosts is very much dependent upon the ability of the parasite to evade the immune system of its host. The ability of *T. brucei* to evade its host immune system is in turn dependent upon the expression of surface coat proteins. The requirement of the *T. brucei* surface coat proteins for fatty acids (FAs) as part of their membrane anchor creates a high demand for FAs in this parasite.

T. brucei readily acquires FAs from its hosts. When the host supply is limited, *T. brucei* can synthesize its own FAs using its FA synthesis pathways. Uptake of lipids requires much less energy than FA synthesis, yet aspects of the biology of this parasite involving the regulation of FA synthesis, the sensing of environmental lipids, and the mechanism of regulation, has never been studied in *T. brucei*. Environmental regulation of FA synthesis would allow preferential use of host FA resources, and would only turn on FA synthesis when the supply of

the host is insufficient. The research I presented in this doctoral dissertation has added to our growing knowledge about lipid metabolism in *T. brucei*.

A key substrate for FA synthesis is malonyl-CoA, which is synthesized from acetyl-CoA by acetyl-CoA carboxylase (ACC). Because ACC likely serves as one control point for regulating FA synthesis, I studied the regulation of *T. brucei* ACC (TbACC) in response to environmental lipids. I demonstrated that procyclic form (PF) *T. brucei* regulated TbACC at the protein, activity, and post-translational levels in response to environmental lipids. The green tea catechin, epigallocatechin gallate (EGCG), induced ACC phosphorylation and reduced TbACC activity (Vigueira *et al.*, 2012). However, unlike in PFs, no change in TbACC RNA or protein levels, activity, or phosphorylation was observed in BFs in response to environmental lipids.

Our current model is that in PFs, TbACC was regulated by environmental lipids, where it played a role in resource management: the parasite only synthesized FAs when host lipids are insufficient. We also demonstrated that at least in PFs, TbACC was both positively and negatively regulated by a variety of metabolites. For example, citrate increased TbACC activity, whereas AMP and long chain fatty acyl-CoAs inhibited TbACC activity. In contrast, BF TbACC was not regulated by environmental lipids. Instead, we propose that BF TbACC was always active to support immune evasion strategies, such as upregulation of endocytosis, that require extensive membrane turnover.

As Russell L. Ackoff rightly said, *“The extensive literature addressed to the definition or characterization of science is filled with inconsistent points of view and demonstrates that an adequate definition is not easy to attain. Part of the difficulty arises from the fact that the meaning of science is not fixed, but is dynamic. As science has evolved, so has its meaning. It takes on a new meaning and significance with successive ages.”* My dissertation work established important background on the regulation of *T. brucei* FA synthesis, while raising many questions. I hope my dissertation stimulates future work aimed at understanding various aspects of *T. brucei* lipid metabolism. Key questions that can be addressed in the future are as follows:

What are the phosphorylation sites in TbACC and how does each site contribute to the regulation of TbACC?

We know that ACC lacks canonical phosphorylation sites, but a recent phosphoproteomics study identified three serines in TbACC that were phosphorylated (Urbaniak *et al.*, 2013). It will be interesting to validate these phosphorylation sites and determine if there are additional phosphorylation sites. This information will provide insight into how phosphorylation of TbACC might alter the activity of the enzyme. Finally it will be useful to determine how phosphorylation at each site is regulated and the contribution of each site to overall TbACC activity.

What is/are the TbACC kinases?

My research provided indirect evidence for multiple kinases (PKA, PKC, PKB, and AMPK) that might potentially phosphorylate TbACC. However, no direct TbACC kinase has been identified. Identifying the TbACC kinase(s) will not only help determine the signaling cascade(s) regulating TbACC and FA synthesis, but also can point the way towards the development of new therapeutics. Inducers of these kinases could trigger TbACC phosphorylation, making it inactive. Inactivating TbACC might have therapeutic potential, as we know from earlier studies in our lab that ACC was essential to establish infection and was required for full virulence in mice (Vigueira and Paul, 2011).

How is *T. brucei* sensing environmental lipids?

We observed stage-specific differences in TbACC regulation in the presence of environmental lipids. All environmental regulation was observed in PFs, whereas in BFs, ACC showed constitutive activity. It will be interesting to know how the parasites “read” the environmental lipids and what proteins are involved. TbACC could potentially sense environmental lipids directly through binding of fatty acyl-CoAs. But the changes in TbACC phosphorylation in response to environmental lipids also suggested that a signal transduction pathway may be involved. Finally, it will be useful to determine the underlying mechanisms governing the differences in how each life cycle stage senses environmental lipids.

Is TbACC regulated by other mechanisms, such as acetylation or ubiquitination?

Acetylation of lysine residues has been demonstrated in various metabolic enzymes in human liver (Zhao *et al.*, 2010; Wang *et al.*, 2010). Key enzymes in FA metabolism, such as acetyl-CoA synthetases, are also acetylated (Starai *et al.*, 2002). It will be interesting to know if TbACC is acetylated and what effect acetylation has on TbACC. Perhaps acetylation plays a role in making the decision between FA synthesis and uptake. Acetylation could alter ACC activity by promoting substrate binding, inhibiting substrate binding, or stimulating selective degradation of TbACC by ubiquitination (Xiong *et al.*, 2011).

Although no change in TbACC mRNA levels were observed, PFs did show changes in TbACC protein levels in response to changes in environmental lipids. This regulation could occur through regulated ubiquitination and degradation of TbACC protein. It will be interesting to examine if high lipid conditions activate a ubiquitin ligase that ubiquitylates TbACC leading to its selective degradation.

The work presented in this dissertation contributed new knowledge about FA synthesis and its regulation in the protozoan parasite *T. brucei*. I hope my work will also shed light on the pathobiology of other related organisms, that possess similar pathways to regulate FA synthesis. An increased understanding of the role of FA synthesis and regulation in host adaptation and pathogenesis opens the door not only to the treatment of HAT, but also may help in the

development of therapeutic approaches against other trypanosomatids such as *Trypanosoma cruzi*, which causes Chagas disease, and *Leishmania*, which causes leishmaniasis.

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