Identification of non-procyclin molecules expressed by *Trypanosoma brucei brucei* procyclic culture forms

by

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Abstract

African trypanosomes cause sleeping sickness in humans and nagana in cattle, both economically devastating diseases in Sub-Saharan Africa, for which a vaccine remains elusive. Trypanosomes exhibit a complex, digenetic life cycle and alternates between the tsetse fly vector (procyclic form) and a mammalian host (bloodstream form). It has proven difficult to study less abundant surface molecules expressed by African trypanosomes because procyclins and/or variant surface glycoproteins (VSG) form a physical barrier that prevents antibodies and labeling reagents from binding to invariant, membrane-embedded molecules. Creating a VSG knock out in bloodstream form trypanosomes has not been accomplished. However, procyclin knock out mutants have been generated that do not express the major surface-disposed procyclins. Other mutants have been generated that do not express any GPI-anchored proteins on their surface. Analysis of protein profiles by 1D gel and 2D gel electrophoresis of wild type, KO1 (*EP/GPEET* knock out), Nour 6 (*EP* knock out) and KO2 (*GPI10* knock out) procyclic culture forms of *T. b. brucei* revealed no differences between the proteins expressed by these parasites. Monoclonal antibodies (mAbs) were generated against the two procyclin knock out mutants, KO1 and Nour 6. After preliminary screening of hybridoma supernatants for mAbs that recognized surface antigens, mAbs 1G10 and 2E1 (anti-KO1), and mAbs 1C3 and 2G4 (anti-Nour 6) were selected for further analysis using immunofluorescence microscopy, flow cytometry and immunoblotting. Using 2D gel electrophoresis and immunoblot analysis in combination with MALDI-TOF mass spectrometry, the antigens recognized by mAb 1G10 were identified as α- and β-tubulin from *T. b. rhodesiense*. MAbs 2E1, 1C3 and 2G4 did not show immunoblot activity. Various attempts to identify the antigens were unsuccessful. However, experiments using the KO2 mutant which, does not express any GPI-anchored surface molecules, allowed tentative identification of some of the antigens as potential GPI-anchored surface molecules. Surface biotinylation with Sulfo-NHS-biotin of wild type, KO1, Nour 6 and KO2 procyclic culture forms of *T. b. brucei* indicated that in addition to the procyclins

these parasites express different surface protein profiles. Biotinylation, in combination with avidin affinity chromatography, was used to isolate surface antigens expressed by KO1 knock out PCF trypanosomes. Two dimensional gel electrophoresis and MALDI-TOF mass spectrometry were used in attempts to identify these surface molecules. The results identified α- and β-tubulin from *T. b. rhodesiense*, in addition to paraflagellar rod proteins, a putative coatomer β-subunit, a probable adenylate/guanylate cyclase and an unknown hypothetical protein from *T. b. brucei*.

Supervisor: Dr. T. W. Pearson, (Department of Biochemistry and Microbiology)

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List of Abbreviations

AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSF	bloodstream form
1D	one dimensional
2D	two dimensional
3D	three dimensional
cAMP	cyclic adenosine monophosphate
CHAPS	(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)
DFMO	difluoromethylornithine
DMSO	dimethyl sulfoxide
DTT	dithiolthreitol
ELISA	enzyme linked immunosorbent assay
EP	glutamate-proline
ER	endoplasmic reticulum
ES	expression site
ESAG	expression site associated gene
FBS	fetal bovine serum
FP	flagellar pocket
GlcNAc	N-acetylglucosamine
GPEET	glycine-proline-glutamate-glutamate-threonine
GPI	glycosylphosphatidylinositol
GPI10	third mannosyl transferase gene
GPI8	protein transamidase gene
GPI-PLC	GPI-specific phospholipase C
GPI-PLD	GPI-specific phospholipase D
HCl	hydrochloric acid
HDL	high density lipoprotein
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRPO	horseradish peroxidase
IgG	immunoglobulin isotype G
IgM	immunoglobulin isotype M
ISG	invariant surface glycoprotein
kDa	kiloDalton
KO1	EP/GPEET procyclin knock out *T. b. brucei*
KO2	GPI10 knock out *T. b. brucei*
LDL	low density lipoprotein
mAb	monoclonal antibody
MALDI-TOF	matrix assisted lazer desorption ionization-time of flight
MSP	major surface protease
NGO	non-governmental organization
Nour 6	EP procyclin knock out *T. b. brucei*
NHS	N-hydroxysuccinimidyl

OD	optical density
PBS	phophate buffered saline
PCF	procyclic culture form
PEG	polyethylene glycol
PIC	protease inhibitor cocktail
PMSF	phenylmethanesulfonyl fluoride
pNNP	p-nitrophenyl phosphate
PVDF	polyvinylidene fluoride
RNAi	ribonucleic acid interferance
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TIFF	tag image file format
THT	trypanosome hexose transporter
TLF	trypanosome lytic factor
Tf-R	transferrin receptor
VSG	variant surface glycoprotein
WHO	world health organization

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Chapter 1. General introduction

I. African trypanosomiasis: the disease

Two subspecies of African trypanosomes cause two different clinical forms of sleeping sickness in humans (Barrett *et al.*, 2000). In West Africa, *Trypanosoma brucei gambiense* is the causative agent of a chronic form of the disease. In contrast, in East Africa, *Trypanosoma brucei rhodesiense* causes an acute form of the disease (Barrett *et al.*, 2000). African sleeping sickness consists of a two stage pathological evolution and, if left untreated, both the acute and chronic forms of the disease are fatal (Buguet *et al.*, 2001). Trypanosomes induce an inflammatory reaction at the site of infection and a skin chancre develops that resolves within one month (Vickerman, 1993). This is the haemolymphatic stage I phase in which the parasites quickly disseminate to the lymphatic system and bloodstream, spreading throughout the body (Vickerman, 1985; Buguet *et al.*, 2001). This early stage is characterized by general malaise, waves of fever, joint pain, peripheral oedema, anemia, headaches and swollen lymph nodes in the neck (Barrett *et al.*, 2000; Burchmore *et al.*, 2002). This can be accompanied by myocarditis, pulmonary oedema, pericardial effusion, ascites, splenomegaly and hepatomegaly (Burchmore *et al.*, 2002). Continuous waves of parasitemia provoke inflammatory reactions within various tissues and organs that have been invaded by the parasites. Stage I ends when the parasites invade the spinal cord and brain. Progression to the neurological stage II phase usually occurs within the first month of a *T. b. rhodesiense* infection but may take several months or years to develop in a *T. b. gambiense* infection (Barrett *et al.*, 2000; Buguet *et al.*, 2001; Burchmore *et al.*, 2002). Stage II can be diagnosed by the presence of parasites

and/or mononuclear inflammatory cells in the cerebrospinal fluid (Barrett *et al.*, 2000; Buguet *et al.*, 2001). Parasite invasion of the central nervous system (CNS) occurs via an unknown mechanism and is accompanied by lymphocyte infiltration, associated vasculitis and perivascular cuffing (Buguet *et al.*, 2001; Burchmore *et al.*, 2002). The disease now manifests itself by causing severe headaches, personality changes, sleep disorders and weight loss, eventually leading to brain function deterioration, seizures, coma and ultimately, death (Black *et al.*, 2001; Burchmore *et al.*, 2002; Stich *et al.*, 2003).

In addition to their great impact on human health, African trypanosomes also cause disease in livestock including cattle and other ruminants, pigs, horses and dogs. The pathogens of animal trypanosomoses are *Trypanosoma brucei brucei, Trypanosoma vivax* and *Trypanosoma congolense* which cause disease in cattle (nagana, from the Zulu meaning "poorly"), *Trypanosoma simiae* which is responsible for high mortality in pigs. In addition to causing disease in cattle *Trypanosoma brucei brucei* affects all livestock, with a particularly high mortality incidence in horses and dogs. Nagana has had a huge impact on agricultural development and socio-economic growth in endemic countries. This is mainly due to the critical role of livestock, primarily cattle, in farming throughout Sub-Saharan Africa (Aksoy *et al.*, 2003).

i. A brief history of African sleeping sickness

Hundreds of years passed between the recognition by slave-traders in West Africa that slaves with swollen lymph nodes at the back of their necks would likely die on the crossing to America, and identification of the parasites that caused this disease. At the

beginning of the twentieth century, the identification of trypanosomes as the causative agent of sleeping sickness was surrounded by controversy. In 1903 Aldo Castellani, working in Uganda, observed a parasite in the cerebral spinal fluid of a patient. However, he did not initially claim this parasite to be the cause of sleeping sickness. David Bruce thought differently and supposedly (as he claimed after the publication) he was the one who convinced Castellani that the parasite might indeed be the culprit. Castellani was given official credit as the sole publisher of this discovery and in the same year Bruce identified the tsetse fly as the active vector (Stebeck *et al.*, 1994) .

Trypanosomes were to play an integral role in the shaping of Africa and its colonization. A description of sleeping sickness dates as far back as the fourteenth century when Ibn Khaldoun in his "History of the Berbers" wrote that the Sultan Djata of the Kingdom of Melli (now Mali) was stricken by a wasting lethargy disease that killed him. Caravanners recognized signs of sleeping sickness in travelers from the southern kingdoms long before they knew the origin or cause. It was not until the twentieth century that particularly severe epidemics occurred in Africa, killing millions and stimulating thorough and extensive control campaigns in afflicted areas. At the turn of the century, from 1896 to 1906, a massive epidemic raged in Uganda and the Congo basin, leaving half a million dead. The second major epidemic was during the 1920's and the third began in the 1990's and continues today (WHO, 2005).

During the second major epidemic the disease was rampant throughout east, south and West Africa. By this time the relationship between trypanosomes, the tsetse fly vector and sleeping sickness had been discovered and drugs were developed to treat the disease. The Sleeping Sickness Bureau, involving multiple countries, pulled together to

initiate a campaign to bring the disease under control. This included treatment of patients and vigilant, continuous screening to decrease the size of the human reservoir that was responsible in part for the rapid transmission. In addition, methodical clearing of undergrowth from villages, water sources, paths and bridges was performed to decrease the natural habitat of the tsetse fly. With the continuous screening of millions of people each year in endemic areas, the number of new cases per year fell and by 1960 the disease was basically eliminated. It took nearly half a century to bring down the disease. Active screening was then largely discontinued and within the last two decades Africa has seen a reemergence of the disease (http://www.who.int/emc/diseases/tryp).

ii. Disease prevalence and its impact today

African independence began within an era of greatly reduced or complete absence of sleeping sickness. However, many of the newly formed countries did not have the human or financial resources to keep up the critical monitoring required to keep the disease in check. Along with the discontinued use and breakdown of disease control programs, and with increased civil unrest, war, famine and the emergence of HIV, it is not surprising that a new epidemic of sleeping sickness began in the 1970's, reached severe epidemic proportions in the 1990s and continues today. African sleeping sickness is now considered a major health problem in sub-Saharan Africa.

African sleeping sickness threatens 60 million people within 36 countries in sub-Saharan Africa **(Figure 1)** (Aksoy *et al.*, 2003). Only three to four million people in endemic areas are under active surveillance and therefore the numbers reported do not reflect the reality of the situation. It is estimated that the number of people currently

afflicted with the disease is between 300,000 and 500,000. In Angola, Central African Republic, Sudan and the Democratic Republic of Congo current epidemics rage with a prevalence of 20-50% making sleeping sickness the leading cause of mortality (ahead of HIV/AIDS) in these areas **(Figure 1)** (http://www.who.int/emc/diseases/tryp).

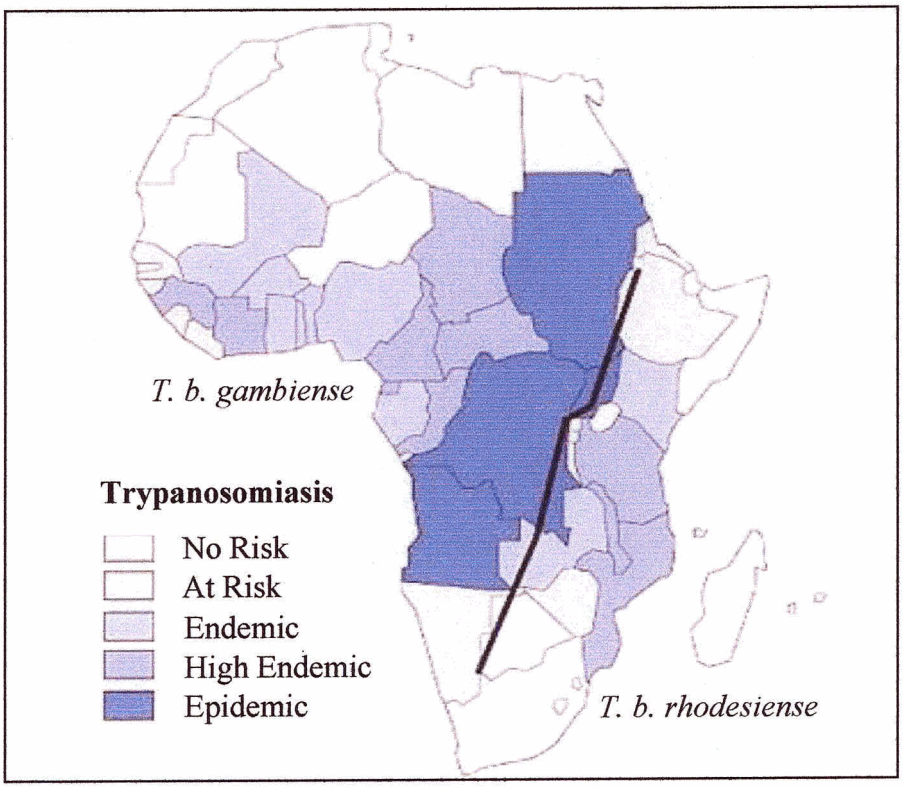

Figure 1. Geographical distribution of African sleeping sickness. *T. b. gambiense* causes chronic disease in West and Central Africa and *T. b. rhodesiense* causes acute disease in East Africa. This is illustrated by the black line that separates sub-Saharan Africa into the two different parasite zones. The legend depicts a scale that goes from areas that have no risk of disease to areas experiencing an epidemic (WHO 2004). (http://www.medicalecology.org/diseases/d_african_trypano.htm)

Of the African trypanosomes that cause nagana in livestock, *T. vivax* and *T. congolense* are the most important and are responsible for massive economic losses, ~ US$ 1338 million annually (Davila, 2003). There are 200 million cattle at risk within sub-Saharan Africa (Aksoy *et al.*, 2003). The trypanosomes are not only critical

economically but are socially devastating as well, causing a decrease in the labor force and abandonment of fertile land where the disease is rife (Stebeck *et al.*, 1994).

iii. Geographical distribution and Transmission

Except for *T. vivax,* which can be mechanically spread by biting insects, and *T. equiperdum*, which is sexually transmitted in horses, transmission of most trypanosomes is strictly dependent upon the tsetse fly insect vector. Consequently, the trypanosomes are restricted to sub-Saharan Africa between the latitudes of 14 °N and 29 °S where the tsetse fly population thrives **(Figure 1)** (Ruepp *et al.*, 1997).

Transmission occurs mainly through the bite of an infected tsetse fly, although it is also possible to contract the disease from contaminated blood, shared needles and in-utero acquisition through the placenta (http://www.who.int/emc/diseases/tryp). There are 31 members of the *Glossina* family and both male and female tsetse flies can transmit the disease (Aksoy *et al.*, 2003). There are seven species of tsetse fly that transmit human sleeping sickness and they are all unique to sub-Saharan Africa. Three *Glossina* species, all of the *palpalis* group, act as vectors for *T. b. gambiense*: *G. palpalis, G. fuscipes* and *G. tachinoides*. Four *Glossina* species of the *morsitans* group are vectors for *T. b. rhodesiense*: *Glossina morsitans morsitans, G. morsitans centralis, G. swynnertoni* and *G. pallidipes*. Tsetse flies require warm, shady, and humid areas to survive and reproduce, therefore restricting the disease to areas in sub-Saharan Africa where these particular conditions can be found. A tsetse fly usually lives for six months and once infected by trypanosomes, remains a vector for life. Tsetse flies that carry *T. b. rhodesiense* live primarily in the savannah woodlands of eastern and southern Africa and

come in contact with humans going about their daily activities such as gathering wood, hunting, fishing, farming and herding cattle. *T. b. gambiense* causes sleeping sickness mainly in lowland rain forests of West and Central Africa and is spread primarily by peri-domestic tsetse flies living in areas surrounding human habitats, such as cultivated land and near small rivers or pools of water, frequented by people. Therefore it is easy to see why this disease is so readily and widely spread (http://www.who.int/emc/diseases/tryp).

II. Diagnosis and Chemotherapeutic treatment of African sleeping sickness

i. Diagnostic tests for African sleeping sickness

Traditional diagnosis was performed using microscopic analysis to visualize trypanosomes in the blood and other body fluids such as cerebrospinal fluid. However, this is unreliable due to the fluctuating numbers of parasites seen throughout the infection (WHO, 1979). Anti-trypanosome antibodies found in the serum are often more reliable, although an active infection, and a cured infection, cannot be distinguished (WHO, 1981). The indirect immunofluorescence antibody test has been the most commonly used diagnostic tool in human sleeping sickness surveys. For this test, blood is collected and eluted from filter paper and used for detection of invariant antigens in acetone-fixed trypanosome smears. Another widely used test for the Gambian form of sleeping sickness is the card agglutination trypanosomiasis test. For this test, a drop of fresh blood is mixed on a card with a drop of fixed, stained trypanosome suspension. A positive test will show macroscopic agglutination within two minutes. Monoclonal antibodies have been developed that distinguish between cattle infections with *T. brucei* sspp, and with *T.*

congolense and *T. vivax* (Vickerman, 1993). More recently, an indirect enzyme-linked immunosorbent assay (ELISA) was used to detect titers of anti-*T. brucei* antibodies in blood collected from primates (Jeneby, 2002). A new form of ELISAs called PCR-ELISAs using species-specific primers has been used to identify *T. vivax* or *T. brucei* infections in cattle (Masake, 2002). Diagnostics are much needed to distinguish between the different stages of disease, as the stage determines the type of treatment. Recently, a test has been devised to identify the stage II forms of disease using IgM intrathecal detection to identify CNS involvement. A latex agglutination test has also been developed which is more practical to use in the field (Lejon, 2002).

ii. Chemotherapeutics for African sleeping sickness

There are no vaccines for African trypanosomiasis; therefore drugs remain the principle means of intervention. Over the past century, there have only been four drugs developed to treat African sleeping sickness, although in the past few years several new ones have been identified and are now in clinical trials. With the standard drugs, there are several problems including: toxicity, increasing drug resistance, administration difficulties, and expense. The drug of choice depends on the infective sub-species of *T. brucei* and whether or not the disease is diagnosed prior to invasion of the central nervous system. The four drugs are: 1) Suramin, 2) Pentamidine, 3) Melarsoprol and 4) Difluoromethylornithine (DMFO).

1) Suramin was first introduced in 1922 and is a polyanionic sulfonated naphylamine, chemically related to Paul Ehrlich's trypan red and other similar naphylamine dyes with trypanocidal activity (Barrett *et al.*, 2000; Denise *et al.*, 2001;

Burchmore *et al.*, 2002; Fairlamb, 2003). Today it remains the treatment of choice for early stage *T. b. rhodesiense* infections, despite its multitude of side effects. These include: nausea, vomiting, rash, fever, shock, collapse and renal damage (De Koning, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003). No significant resistance to the drug has emerged within the last 80 years. However, it has a 25-35% treatment failure rate (Burchmore *et al.*, 2002; Fairlamb, 2003). Its mode of action remains obscure even after many years of investigation. Suramin contains six negative charges at physiological pH and therefore it has a high avidity for serum proteins including low density lipoprotein (LDL) (Barrett *et al.*, 2000; De Koning, 2001; Denise *et al.*, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003). Trypanosomes have a receptor for low density lipoprotein (LDL) and it has been hypothesized that LDL is used to accumulate the drug via receptor-mediated endocytosis, although this has recently been disputed (Denise *et al.*, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003).

2) Pentamidine was discovered after a similar compound, (synthalin), which induces hypoglycaemia in mammals, was found to have profound anti-trypanosomal activity (Denise *et al.*, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003). This drug has been in use for over 50 years and was introduced in the 1940's as a trypanocidal chemotherapeutic (Burchmore *et al.*, 2002; Fairlamb, 2003). Treatment is restricted to the early stage of *T. b. gambiense* infections because the drug does not readily cross the blood-brain barrier to the central nervous system. It is not understood why it fails with *T. b rhodesiense* infections (Barrett *et al.*, 2000; Denise *et al.*, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003). Side effects include: nausea, severe hypotensive reactions following injection, liver and kidney damage (nephrotoxicity) and pancreatic damage, which can

lead to diabetes (Burchmore *et al.*, 2002; Fairlamb, 2003). This drug has poor oral bioavailability and must be administered by peritoneal injection making it difficult to administer in the field. Despite broad use of this drug in attempts to eradicate disease caused by *T. b. gambiense* during the 1950's and 1960's, widespread resistance has not emerged in the field (Fairlamb, 2003). The multiple means by which pentamidine is taken up by the parasite may account for the lack of drug resistance (Barrett, 1999; De Koning, 2001; De Koning, 2001; Denise *et al.*, 2001; Fairlamb, 2003). Pentamidine is an aromatic diamidine that acts directly against the parasites, independent of their physiological action on the host (Barrett, 1999; Barrett *et al.*, 2000; De Koning, 2001; De Koning, 2001; Denise *et al.*, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003). The mechanism of action is not well understood, although it is known to be taken up by at least three transporters (P2, HAPT1 and LAPT1) and accumulated to millimolar concentrations within the cells (Barrett, 1999; Hanna, 2000; De Koning, 2001; De Koning, 2001; Denise *et al.*, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003).

3) Melarsoprol was developed based on information gained from studies of arsenicals, first introduced by Paul Ehrlich at the turn of the century (Barrett *et al.*, 2000; Denise *et al.*, 2001; Burchmore *et al.*, 2002). Melarsoprol was introduced to the market as an anti-trypanosomal drug in 1949. It is the only drug available to treat late stage *T. b. gambiense* and *T. b. rhodesiense* infections and resistance has been reported in the field (Barrett *et al.*, 2000; Denise *et al.*, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003). Melarsoprol is water insoluble and must be given intravenously dissolved in propylene glycol, a highly irritable solvent (Fairlamb, 2003). Of all the trypanocidal drugs, melarsoprol causes the most severe toxic effects including encephalopathy in 5-10% of

cases, of which half are fatal (Burchmore *et al.*, 2002; Fairlamb, 2003). Other common side effects include: vomiting, abdominal colic, peripheral neuropathy (seizures), arthralgia, and thrombophlebitis (leakage of propylene glycol from the site of injection into surrounding tissue) (Burchmore *et al.*, 2002; Fairlamb, 2003). The mechanism of the severe and potentially fatal encephalopathy is unknown but does not seem to be restricted to patients treated for sleeping sickness (Fairlamb, 2003). Trivalent arsenicals are promiscuous inhibitors of many enzymes or substrates that contain vicinal thiol groups. Trypanosomes express a specific purine transporter and loss of this transporter leaves trypanosomes resistant to Melarsoprol. Once within the cell, the arsenical drug could potentially inhibit many vital metabolic and transport functions, which could lead to loss of motility and lysis which parasites experience when treated with these drugs (Burchmore *et al.*, 2002; Fairlamb, 2003).

4) DMFO was first developed as an anti-cancer reagent. However, even though it remains at the trial stage for treatment of neoplastic disease, it does exhibit activity against both early and late stage *T. b. gambiense* sleeping sickness (Barrett *et al.*, 2000; Denise *et al.*, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003). Most cases do not respond effectively and therefore DFMO is not used for treatment of *T. b. rhodesiense* sleeping sickness (Denise *et al.*, 2001; Fairlamb, 2003). This drug is far from ideal as it is very costly ($750/patient) and difficult to administer because it requires four daily infusions of 400 mg/kg/day over a 2 hour period for 7-14 days (Barrett *et al.*, 2000; Burchmore *et al.*, 2002; Fairlamb, 2003). Side effects of DFMO are minimal, however diarrhea, anemia and other blood cell reductions are known. The lack of toxic side effects is due to the mechanism by which this compound elicits its trypanocidal activity. DFMO is an analog

of ornithine and acts as a specific, irreversible inhibitor of the enzyme ornithine decarboxylase (ODC), the first committed step in the biosynthetic pathway of polyamines. DFMO has similar specificity for both parasite and mammalian ODC. However, mammalian cells are able to replenish ODC much faster than trypanosomes, therefore a pulse of DFMO can deprive trypanosomes of ODC for a long period of time compared to mammalian cells, leading to cessation of parasite growth (Barrett *et al.*, 2000; Denise *et al.*, 2001; Burchmore *et al.*, 2002; Fairlamb, 2003).

iii. Drug resistance and future alternatives for control of African trypanosomiasis

Treatment of sleeping sickness is limited to a very small number of drugs that are expensive, difficult to administer in the field, and toxic. In addition, they are becoming increasingly ineffective due to the emergence of drug resistant parasites. With the resurgence of trypanosomiasis across Africa and the increase in arsenic resistant parasites, the need for new anti-trypanosomal drugs has never been greater. There are huge obstacles to overcome before new drugs are developed and put into use. These include the stringency of licensing criteria that will not allow potential drugs such as megazol to proceed to final trials because it does not pass the Ames test, even though it has cured Simians of trypanosomiasis with a single dose and exhibited no side effects (Denise *et al.*, 2001). Pharmaceutical companies are reluctant to invest in development of new drugs for sleeping sickness because of the unlikely return of their investment from markets in developing countries. This often leaves academia responsible for drug development (Denise *et al.*, 2001; Davila, 2003). Human African sleeping sickness seems to have received a great deal of scientific interest when compared to other

diseases. However, this is misleading because African trypanosomes have become a paradigm for research into basic biology and eukaryotic cell function due to several unique characteristics with respect to their biochemical composition and gene regulation (Stich *et al.*, 2003). Therefore, the bulk of research focused on trypanosomes does not encompass studies on diagnosis, treatment or control (Stich *et al.*, 2003). This paints a bleak picture. However, three initiatives have been put into motion aimed at bringing the disease back under control (Stich *et al.*, 2003). First, the International Atomic Energy Agency (IAEA) announced their plan to implement a continent-wide release of sterile tsetse. This followed a successful trial on Zanzibar where tsetse trapping, insecticide spraying and release of sterile males led to the complete eradication of tsetse from the island (Stich *et al.*, 2003). This goal seems unrealistic, as it cost millions of dollars just to eradicate tsetse from Zanzibar, a small isolated island, whereas the tsetse belt in sub-Saharan Africa covers an area greater than the United States of America. Second, the World Health Organization (WHO) and Médecins Sans Frontieres (Doctors Without Borders) initiated a campaign to encourage two pharmaceutical companies to re-engage in production of drugs for sleeping sickness. This was done after the "DFMO scandal" which occurred when DFMO was no longer produced in the formulation needed for treatment of human sleeping sickness but instead appeared as a facial depilatory cream. After much outcry, Aventis agreed to donate pentamidine, melarsoprol and DFMO in 2001. Bayer then committed to continued production of suramin (Stich *et al.*, 2003). Third, a major funding initiative by the Bill and Melinda Gates Foundation has enabled one compound, the pro-drug DB 289, a dicationic compound, to enter clinical trials (Stich

et al., 2003). The DB 289 compound has proven to be extremely effective and is now entering Phase III clinical trials (http://www.immtech.biz/african.html).

In countries most affected by the resurgence of sleeping sickness (Sudan, Central African Republic, Democratic Republic of Congo and Angola) control options exceed the capacities of the responsible governmental institutions. This is due to a combination of lack of funds, movement of people, famine, drought, civil unrest and/or war which lead to a breakdown of even the most basic infrastructure (Abel *et al.*, 2004). In many places, such as Angola, NGOs have intervened, in an attempt to help bring the disease back under control (Abel *et al.*, 2004). African sleeping sickness is a public health problem in countries where research infrastructure barely exists, hence, the absence of scientific research to answer fundamental questions in the understanding of disease pathogenesis, clinical presentation, effective control approaches, diagnostics and treatments (Abel *et al.*, 2004). The disease, which is often present at very low levels, returns as soon as active surveillance is abandoned, usually due to war, lack of security, and poverty.

It is obvious from the resurgence of sleeping sickness that new control methods are required along with more consistent surveillance of endemic areas. One approach is the screening of new trypanocidal compounds to develop new drugs for treatment. Ideally these would be inexpensive to manufacture, less toxic than current drugs, orally administered and designed to be refractory to biochemical mechanisms responsible for drug resistance. An alternative approach to drugs that target the parasite, is the use of sterile insect technology ie. approaches involving tsetse eradication by release of sterile males. Another approach that is gaining popularity is the genetic engineering of symbiotic bacteria to manipulate vector-parasite interactions. It has been shown that

production of parasite-inhibitory molecules by vector symbionts greatly reduces parasite development and viability within the tsetse fly. This approach is called paratransgenesis. Tsetse flies naturally harbor bacterial symbionts that could theoretically be altered to express a foreign gene product. The primary symbiont *Wigglesworthia glossinidia* lives in specialized cells called bacteriocytes found in the bacteriome whereas the secondary symbiont, *Sodalis glossinidius* lives both freely in the hemolymph and midgut and intracellularly in midgut epithelial cells. The midgut is an ideal compartment to inhibit trypanosome development, differentiation or transmission. In the midgut bloodstream form (BSF) trypanosomes differentiate into procyclic forms, for development and transmission through the vector. Inhibition of either of these steps by a molecule produced by an altered symbiont could greatly affect establishment, differentiation and transmission of the parasite, and therefore decrease disease prevalence. *S. glossinidius* can be efficiently cloned and grown *in vitro* and has already been genetically engineered to produce a trypanocidal cationic peptide attacin, which inhibits growth of both BSF and procyclic forms *in vitro* and *in vivo* (Hu, 2005). This has so far proven to be a very promising therapeutic approach for the future control of trypanosomiasis (Hu, 2005).

III. Trypanosome biology

African trypanosomes are amongst the earliest eukaryotic microorganisms. They are single celled, flagellated protists of the kinetoplastid group of protozoa. These parasites are organisms that complete part of their life cycle in a mammalian host and part in a tsetse fly vector. These organisms have gained notoriety, not only because they

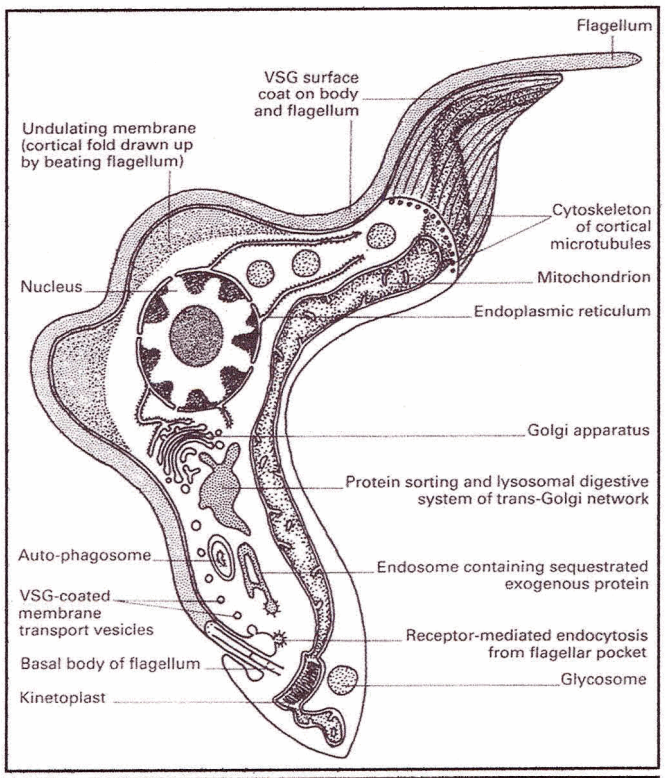

Figure 2. Principal cellular structures of *Trypanosoma brucei* depicted as a partial longitudinal section. (Vickerman, 1993)

cause devastating disease in both humans and animals, but because they have the unique capacity to adapt to a huge variety of environments, illustrating the capabilities of a single cell (Maga, 1999).

i. Parasite molecular biology

At each life cycle stage African trypanosomes can be characterized by the cell shape, size and motility, metabolism and surface coat (Gull, 2001). The parasites have an elongated shape that changes in size from a long slender form in the bloodstream of a mammalian host to the short stumpy forms that establish infection in the tsetse fly vector. The cell shape is conferred by a network of microtubules that underlie the entire plasma membrane, with the exception of a small invagination at one end, termed the flagellar

pocket, from which the flagellum emerges (Bangs, 1998; Maga, 1999). All trypanosomes have a nucleus, rough and smooth endoplasmic reticulum, a single morphologically identifiable Golgi apparatus, small as well as large lysosomal-like vesicles, flagellum, mitochondrion and a specialized peroxisome-like organelle termed the glycosome **(Figure 2)** (Clayton, 1995; Bangs, 1998; Maga, 1999; Cross, 2001; Gull, 2001). African trypanosomes are strictly extracellular pathogens, using their single flagellum for motility. However, they do exhibit some stationary stages in the vector; they form interdigitations with epithelial cell microvilli in the midgut and hemidesmosomal-like attachments on a variety of tissues via their flagellum, more specifically, the paraflagellar rod proteins that extend along the length of the flagellum (Maga, 1999). Kinetoplastids were amongst the earliest organisms to have either a mitochondrion or peroxisome (Clayton, 1995). The nucleus of *T. brucei* contains a genome comprised of 11 diploid megabase chromosomes, a set of intermediate-sized chromosomes and a large number of mini-chromosomes (McCulloch, 2004). Extensive vesicular trafficking is seen between the flagellar pocket and the nucleus where the single Golgi apparatus is situated **(Figure 2)**. The flagellar pocket is the only site of endocytosis and exocytosis in the bloodstream form of the parasite because it is the only site on the cell surface that is not underlined by the microtubular network (Clayton, 1995; Bangs, 1998; Maga, 1999). Endocytosis and exocytosis occur at much higher rates in the BSF, probably due to the high turnover rate of the major variant surface glycoprotein (VSG). The flagellar pocket also seems to be the only area on the cell surface where membrane bound, surface receptors are exposed to the extracellular environment (Pays *et al.*, 1998). The flagellum is anchored in the cell by the basal body which physically interacts with the mitochondrion. The mitochondrion is

a branched network of cristae that enlarges during differentiation into the procyclic stage (Clayton, 1995). A complex network of mitochondrial DNA is concentrated in the region closest to the flagellar pocket and is called the kinetoplast **(Figure 2)** (Clayton, 1995). The kinetoplast consists of a network of thousands of topologically interlocked DNA circles and is a unique structure (Clayton, 1995). There are about 50 copies/cell of 20-40 kb maxicircle DNA and 5,000-10,000 copies/cell of 0.65-2.5 kb minicircle DNA in the kinetoplast (Clayton, 1995; Gull, 2001). Mitochondrial mRNAs undergo a unique form of post-transcriptional modification, termed RNA editing. During this event uridylate residues are inserted and deleted from mRNAs to produce mature mRNA molecules (Clayton, 1995; Cross, 2001; Gull, 2001; Gull, 2003).

African trypanosomes have become very well known for some unique characteristics with respect to their metabolism and surface coat proteins that both change depending on the life cycle stage of the parasite (Bangs, 1998). These include the enzymes required for production of ATP via glycolysis. The seven glycolytic enzymes responsible for converting glucose to 3-phosphoglycerate are housed in a specialized organelle called the glycosome. The other glycolytic enzymes are contained in the cytoplasm where the Krebs cycle and respiratory chain enzymes are also located (Vickerman, 1993; Clayton, 1995; Cross, 2001; Gull, 2003). In the bloodstream, the parasites exploit the glucose rich environment; however, in the tsetse fly midgut proline becomes the main energy source. Throughout the life cycle the parasites express a unique cell surface coat that forms the first line of contact with both the host and vector (Bangs, 1998). In the bloodstream, parasites undergo antigenic variation by replacing their VSG coat to escape the host immune system. The procyclic forms express two

different major forms of procyclins, thought to be involved in protease protection, differentiation and tropism through the vector.

ii. Parasite life cycle in the tsetse fly vector

Trypanosomes exhibit a complex, digenetic life cycle that alternates between the tsetse fly vector and a mammalian host **(Figure 3)**. The life cycle of trypanosomes within the vector begins when a tsetse fly takes a bloodmeal from an infected mammalian host and ingests infective BSF trypanosomes. Differentiation and migration, from the midgut to the salivary glands, completes this part of the life cycle. This can span a period of a few days in *T. vivax* to a few weeks in *T. brucei* sspp. (Aksoy *et al.*, 2003). Once ingested by the fly, the trypanosomes must quickly adapt to a completely new and hostile environment, to do this they undergo morphological and biochemical changes to survive (Treumann *et al.*, 1997). They must transform in order to grow and divide at a lower temperature (27 °C as opposed to 37 °C), utilize a different food source and protect themselves against destructive digestive enzymes and immune molecules (Aksoy *et al.*, 2003). This transformation can be induced *in vitro* by triggering the citric acid cycle via the addition of cis-aconitate or citrate (Vassella *et al.*, 2003). However, it is thought that these metabolites are not involved *in vivo* (Vassella *et al.*, 2001; Aksoy *et al.*, 2003). Other triggers of differentiation include temperature drop, trypsin and mild acid stress. Of these, only the temperature drop is known to be experienced by the trypanosomes *in vivo*, in the fly. The BSF are ingested first into the crop, and then enter the midgut lumen.

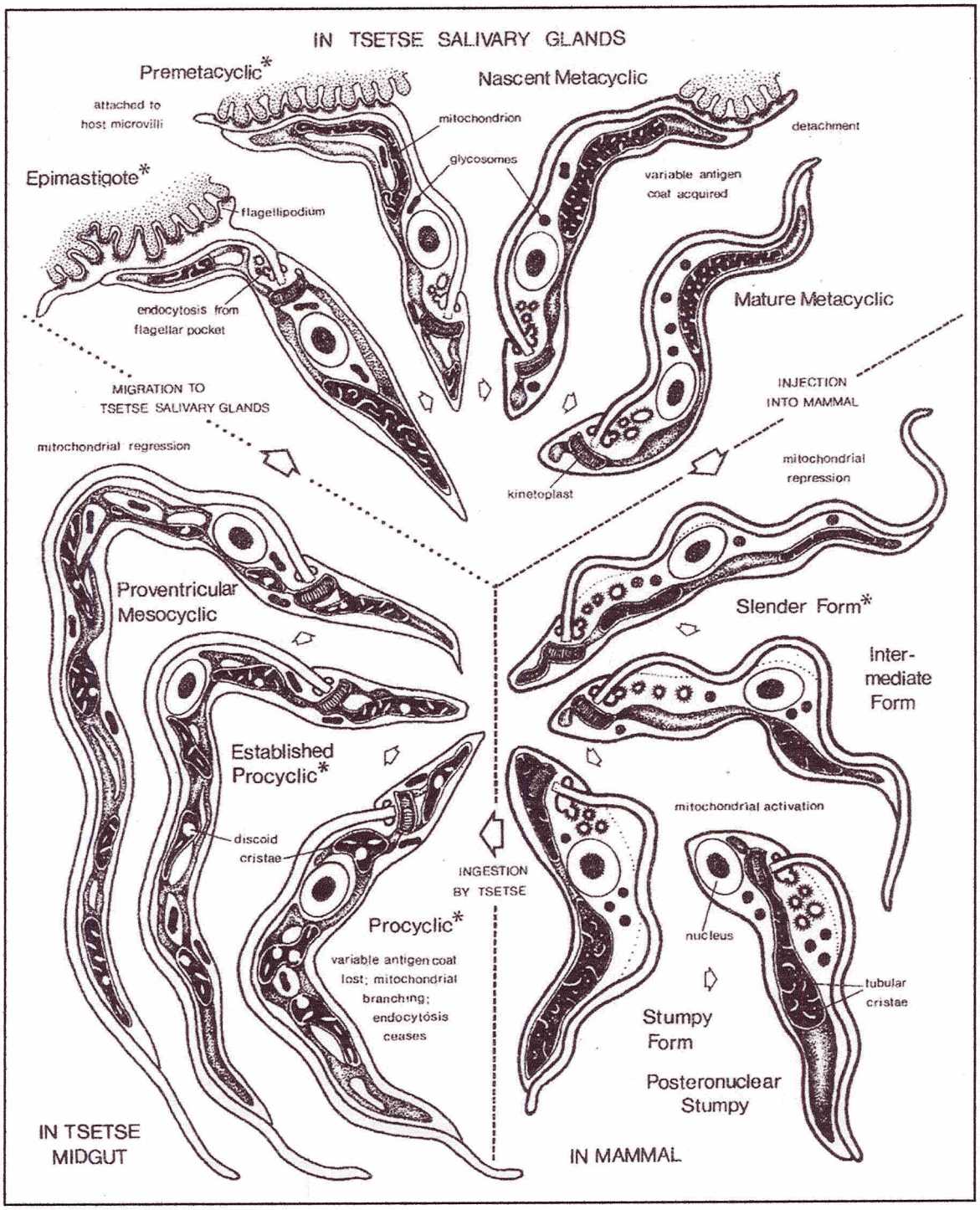

Figure 3. Schematic diagram of the digenetic life cycle of African trypanosomes. The diagram shows changes in cell surface, mitochondrion, glycosomes, receptor-mediated endocytosis and the relative size of the different stages. The VSG coat is shed concurrently with the gain of procyclins upon entry into the tsetse midgut. VSG is regained in the metacyclic stage in the salivary gland. Division occurs in the slender form in the mammal and the procyclic form in the tsetse midgut (Vickerman, 1985).

slender BSF usually die and the short-stumpy forms differentiate into procyclic trypanosomes in endoperitrophic space of the midgut (Vickerman 1985; Vickerman; 1993). The trypanosomes undergo critical transformations within the first 24-72 hours, in order to survive and develop in their new host (Vickerman, 1985). The most apparent change is the loss of the VSG surface coat and its successive replacement with procyclins. The transforming trypanosomes increase in body length, while the mitochondrion expands into a branched network with discoid rather than tubular cristae and increases in size by 5% to 25%. Concomitantly, the glycosomes change from spherical shapes to bacilliform structures and endocytosis ceases **(Figure 3)**. This is all accompanied by rapid division of the flagellates (Vickerman, 1985). Biochemically, the procyclic forms switch from using glucose to proline as the primary energy source. Activation of the mitochondrion is also associated with a switch to cytochrome-mediated terminal respiration and this may correlate with the change in form of the mitochondrial cristae. From four days onwards, the trypanosomes actively invade the ectoperitrophic space between the peritrophic matrix and the gut epithelium (Vickerman, 1985; Acosta-Serrano *et al.*, 2001). Within the ectoperitrophic space, rapid division occurs over the next week and this space becomes densely packed with parasites. As the parasites move forward to the proventricular space they grow very long, (up to 60 μm), cease to divide and are termed mesocyclic trypanosomes **(Figure 3)**. It is thought that these parasites are the ones to reinvade the endotrophic space in order to undergo migration to the salivary glands via the oesophagus, mouthparts and salivary ducts (Vickerman, 1985). Infection is established within the salivary glands, where four different life cycle stages develop. The epimastigotes are the main proliferative stage and become attached to the microvillae

via the flagellum. Attachment appears to be essential for the generation of infective metacyclics. Transformation from the epimastigote to the metacyclic stage occurs via two intermediate stages. The attached epimastigotes lose their procyclin surface coat and transform into premetacyclics. These parasites still divide, and retain the branched mitochondria and bacilliform glycosomes, however, the flagellipodia are greatly reduced. The nascent metacyclic forms cease division and detach from the microvillae. Transformation into mature metacyclics is accompanied by, re-acquisition of the VSG surface coat, unbranched mitochondria and spherical glycosomes. This indicates a change in metabolism, in preparation for survival within the mammalian host **(Figure 3)** (Vickerman, 1985).

The advantages gained by the trypanosome during its life cycle within the fly include: amplification of a typically sparse parasitaemia in the host, the potential transfer of infective forms every time the fly takes a bloodmeal from a new host, and the opportunity for sexual reproduction (Aksoy *et al.*, 2003). Taking the whole life cycle into consideration, the passage through the fly plays a critical role and is essential in maintaining species integrity, apart from being a convenient mode of transmission (Aksoy *et al.*, 2003).

iii. Parasite life cycle in the mammalian host

The trypanosome life cycle within the mammalian host begins when an infected tsetse fly bites and takes a bloodmeal. During this transaction saliva and non-dividing metacyclic trypanosomes from the salivary gland enter into the skin (Vickerman, 1985; Matthews *et al.*, 2004). Trypanosomes induce an inflammatory reaction at the site of

infection, and a skin chancre develops that resolves within one month (Vickerman, 1993). From the initial site of infection the parasites enter the draining lymph nodes and then the bloodstream. They travel through blood vessels and lymphatic capillaries into connective tissue and eventually enter the brain and cerebrospinal fluid (Vickerman, 1985). Within the mammalian host they are subjected to specific and non-specific immune responses and have therefore evolved complex strategies with which to evade eradication from the host (McCulloch, 2004).

The VSG-expressing metacyclic forms must quickly transform into BSF in order to survive within the host (Vickerman, 1985; Matthews *et al.*, 2004). This includes replacing the metacyclic VSGs with BSF VSGs. BSF trypanosomes exploit the abundance of glucose found in the bloodstream of mammalian hosts and this becomes their primary energy source. The bloodstream population is described as pleomorphic for it contains slender forms, stumpy forms, and transitional forms at different intermediate stages between the latter two **(Figure 3)** (Matthews *et al.*, 2004). The transition between dividing, slender forms, and the non-dividing, stumpy forms, requires progression from proliferation to cell cycle arrest. This is accompanied by a number of morphological and biochemical transformations. Key features of the stumpy form include: cell cycle arrest, elaboration of some mitochondrial functions, and relative resistance to antibody-mediated lysis. If a bloodmeal is taken from an infected host it is the stumpy forms that will progress to the next stage in the life cycle within the tsetse fly midgut **(Figure 3)** (Matthews *et al.*, 2004).

African trypanosomes have developed mechanisms to disrupt and inhibit host defenses. Investigation into the mechanisms used by African trypanosomes to evade the

immune responses of their mammalian hosts began in the early nineteen hundreds. Infected mammals produce antibodies to a highly immunogenic surface molecule (Black *et al.*, 2001; Morgan *et al.*, 2002). In 1907 an Italian scientist, A. Massaglia, concluded that African trypanosomes in the bloodstream escaped destruction because they adapted to interfere or block the action of antibodies (Donelson, 1998). In 1975, Cross described heterogeneous surface glycoproteins, expressed by BSF trypanosomes, which induced clone-specific immunity in infected mice (Cross, 1975). This article proved that seventy years earlier Massaglia had come to the right conclusion. This unique technique of switching surface coats to evade host immune responses was termed antigenic variation. BSF trypanosomes have become famous, because much research has gone into elucidating the molecular mechanisms responsible for this phenomenon (Donelson, 1998). Individual trypanosomes express one VSG at a time, therefore the mammalian host experiences recurring waves of parasitaemia because the main immune response is against the VSG which changes with each new wave. A VSG specific response takes many days to develop, therefore the host experiences high levels of parasitemia due to the quick doubling time of trypanosomes (approximately 7 hours). This leads to severe pathology, immunosuppression and eventually debilitating secondary infections in the host (Black *et al.*, 2001).

Antigenic variation is not the sole factor responsible for immune evasion and maintenance of a chronic infection (Black *et al.*, 2001; Morgan *et al.*, 2002). As BSFs are lysed and destroyed throughout an infection the immune system is assaulted not only by different classes of VSGs but also by invariant, intracellular antigens. Parasite levels increase dramatically upon initial infection inducing a massive, non-specific expansion of

polyclonal B-cells. These B-cells produce immunoglobulin isotype M (IgM), antibodies, that recognize parasite antigens, which may also cross-react with self-antigens (Donelson, 1998; Pays *et al.*, 1998). The huge IgM response is not followed by the typical concomitant increase in immunoglobulin isotype G (IgG) and other antibody classes. Suppression of many B-cell and T-cell responses is accompanied by the suppression of other secondary immune events. Fore example, interferon gamma production is increased, and may correlate with increased macrophage activity. Interleukin-2 levels are decreased which in turn may explain the lack of T-cell proliferation. Parasites appear to evade complement-mediated lysis by opsonization and destruction by liver macrophages (Donelson, 1998).

IV. The importance of trypanosome cell surface molecules

The trypanosome surface is the first interface to come in contact with the hostile environment of the mammalian bloodstream or the insect midgut. Therefore, the major surface molecules coating the bloodstream form (mammalian) and procyclic form (insect) trypanosomes are the first line of defense against the host immune system. The major surface glycoproteins of BSF and procyclic form, likely play a role in establishment of infection, parasite development and differentiation. In addition to the major surface glycoproteins, less accessible, underlying surface molecules are also present, and may provide essential functions for parasite viability. The critical role surface molecules play in the life cycle and parasitaemia of African trypanosomes makes these molecules attractive research subjects. Chapter 2 will discuss the major and minor surface molecules expressed by African trypanosomes that are known today. What follows is a

molecular and biochemical study of the surface proteins expressed by wild type PCF *T. b. brucei* and three knock out PCF mutants that differ in their surface protein expression.

Chapter 2. The surface coat of PCF African trypanosomes

1. Introduction

The surface coat of African trypanosomes is comprised of two major surface proteins. As trypanosomes move between hosts in their life cycle and go through their different developmental stages the expression of these two surface molecules changes as previously discussed in Chapter 1. Chapter 2 begins with a closer look at the structure and regulation of these surface molecules.

1.1 Variant surface glycoproteins of bloodstream forms of African trypanosomes

i. Regulation of VSG gene transcription

Bloodstream forms of African trypanosomes are infamous for their ability to show antigenic variation which allows them to avoid immune elimination. In a population of parasites, there is sequential expression of individual members of a large repertoire of variant surface glycoprotein (VSG) genes that encode antigenically distinct proteins (Donelson, 1998).

The trypanosome genome contains several hundred genes encoding immunologically distinct VSGs. To ensure that an individual cell expresses only one VSG, there are multiple sites of transcription, and only one is actively transcribed at a time (Ferguson, 1997). Large numbers of silent *VSG* genes are also spread throughout the genome. There are tens of *VSG* expression sites, and hundreds of silent *VSG* genes (McCulloch, 2004). There is strong evidence to indicate that the general pathways of

homologous recombination and repair are responsible for *VSG* switching. It is thought that a gene copy is generated from a silent *VSG* gene and then moved into an active expression site (ES), deleting the resident *VSG* gene and leaving the formerly silent one to be expressed (Pays *et al.*, 1998; McCulloch, 2004). Within the mammalian bloodstream, *T. brucei* expresses a class of *VSG* expression sites called BSF ESs, which have complex structures containing Expression Site-Associated Genes (ESAGs) that are co-transcribed along with the *VSG* gene. In the salivary gland, where trypanosomes differentiate into infective metacyclics, a class of *VSG* genes called telomeric metacyclic ESs are active so that the parasite can reacquire its VSG coat (McCulloch, 2004).

ii. Structure of VSG proteins

Bloodstream forms express an unusual surface coat composed of about one million copies of VSG glycoproteins arranged as a dense monolayer of homodimers on the parasite surface **(Figure 4)** (Ferguson, 1997; Mehlert *et al.*, 1998; Pays *et al.*, 1998). Each VSG molecule has an approximate mass of 55 kDa. The amino terminal domain of 350 to 400 residues represents about 75% of the polypeptide and is highly variable (Mehlert *et al.*, 1998; Pays *et al.*, 1998). However, there is some sequence similarity amongst VSG N-terminal domains implying that some structure is conserved. X-ray crystallography studies showed that different VSG polypeptides fold into similar elongated shapes, with two extended anti-parallel alpha-helical bundles per monomer forming a 15 nm thick coat **(Figure 4)** (Clarke, 1988; Ferguson, 1997; Mehlert *et al.*, 1998; Pays *et al.*, 1998). In 1988 Clarke and colleagues showed that the antigenic

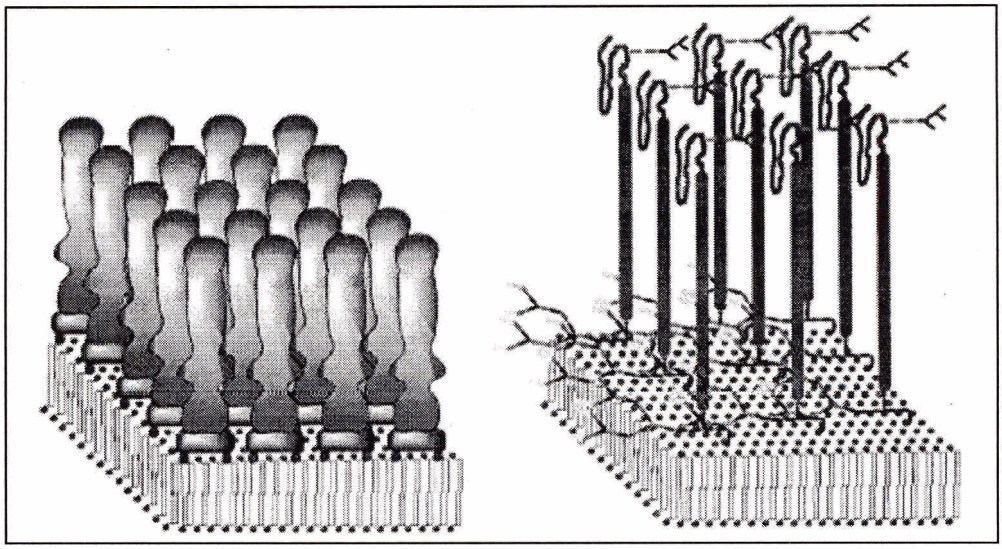

Figure 4. Simple model of the major surface molecules of bloodstream form and procyclic form *T. brucei*. VSGs form a dense surface coat on BSF in comparison to the more diffuse surface coat of PCF comprised of procyclins. VSG dimers sit on top of a glycocalyx made up of the glycans and GPI anchors. Procyclins form rod like structures attached to the membrane via GPI anchors that are highly glycosylated. (Mehlert *et al.*, 1998)

differences between VSGs were dependent on topographical assembled epitopes on the parasite surface of epitopes that were highly sensitive to structural changes (Clarke, 1988). All VSGs are post-translationally N-glycosylated, typically once or twice, at or near the C-terminal domain (Ferguson, 1997; Pays *et al.*, 1998). All VSGs are attached to the plasma membrane through a covalent linkage between the C-terminal amino acid and a glycosylphosphatidylinositol (GPI) membrane anchor (Pays *et al.*, 1998). Mature VSG GPI anchors contain a common core structure of ethanolamine-HPO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6PI characteristic to most GPI anchors. However, this anchor is then modified by a galactose side chain that is unique to VSGs. The 3-D structure predicts that the anchor forms a large, dense, plate-like calyx of carbohydrate on which the VSG polypeptide sits (Ferguson, 1997).

iii. Functions of VSG

The VSG proteins are so densely packed that a very limited stretch of N-terminal amino acid sequence is accessible to the extracellular environment and all of the C-terminus is buried (Mehlert *et al.*, 1998; Pays *et al.*, 1998). The dense packing combined with the overall thickness of the VSG coat means that only a limited subset of B-cell-stimulating epitopes are exposed on the surface (Ferguson, 1997; Pays *et al.*, 1998; McCulloch, 2004). VSGs are highly immunodominant and the mammalian host recognizes and mounts an immune response to these proteins. Therefore the VSG coat shields underlying invariant surface proteins that would be potential targets for attack by the immune system (Pays *et al.*, 1998). Consequently, antigenic variation of VSGs and their densely packed surface expression enables some parasites to evade both specific humoral immune responses as well as components of the alternative complement pathway (Mehlert *et al.*, 1998). VSG switching occurs at a frequency of 10^{-2} per cell and per generation so that the parasites can maintain a persistent infection rather than be completely eliminated (Pays *et al.*, 1998; McCulloch, 2004).

1.2 Procyclins expressed by PCF African trypanosomes

Although many laboratories have been engaged in research to develop a vaccine against BSFs of African trypanosomes, this has proved frustrating, and to date, futile. Consequently, some research groups have become more interested in studying the procyclic form of the parasite and tackling the biology of parasite-vector interactions. The interaction between parasite and host molecules within the tsetse fly vector are vital, and offer potential control strategies that could limit or halt transmission of the disease.

i. The discovery of procyclins

The surface of procyclic form trypanosomes was long thought to be coatless because the VSGs were lost upon transformation of BSF to procyclic culture forms (procyclic forms adapted to *in vitro* growth) (Roditi, 1990). The discovery of procyclins lagged two decades behind that of VSG mainly because procyclins could not be detected by several procedures typically used for identification of proteins (Richardson, 1988; Roditi, 1990). They could not be iodinated, nor biosynthetically labeled by [^{35}S]methionine, did not stain with Coomassie blue and were not detectable by absorption at 280 nm. Therefore they were biochemically invisible, at least by several of the most commonly used techniques (Roditi, 1990; Roditi *et al.*, 1998). Eventually these molecules were discovered by two different approaches and two different investigative teams that ultimately converged and complemented each other (Roditi, 1990). One approach involved derivation of monoclonal antibodies against procyclic culture forms (PCF) and then purification of the glycoprotein (Richardson, 1986; Richardson, 1988), and the second involved identification of a cDNA clone that hybridized specifically to mRNA transcripts from PCF but not BSF (Roditi, 1987). The nucleic acid sequence of these transcripts predicted that membrane-bound glycoproteins containing extensive glutamic acid-proline repeats were expressed only in PCF and not BSF (Roditi, 1990; Treumann *et al.*, 1997). Due to collaboration between the Roditi and Pearson laboratories, which included the exchange of materials, the procyclic surface molecules of *T. b. brucei*, termed procyclins, were identified. The name procyclin stems from their stage specific expression in procyclic parasites and from their high proline content

(Richardson, 1988; Roditi, 1990). Thus the procyclic form trypanosomes are not-so-naked after all (Roditi, 1990).

ii. The structure of procyclins

Procyclic forms express a surface coat that could be described as a dense GPI glycocalyx immediately adjacent to the membrane, from which protrude the highly extended polyanionic rod-like structures of the procyclin polypeptides **(Figure 4).** The procyclins of *T. brucei* sspp. include two major classes of proteins that contain distinctive internal amino acid repeats. In total, approximately 2.2 X 10^6 copies per cell are expressed on the surface of PCF. EP procyclins contain 22-30 internal Glu-Pro (EP) dipeptide repeats and GPEET procyclin contains six Gly-Pro-Glu-Glu-Thr (GPEET) repeats followed by three EP repeats **(Figure 5)** (Vassella *et al.*, 2001; Roditi *et al.*, 2002). There are three EP procyclin genes: *EP1, EP2* and *EP3,* and one GPEET procyclin gene: *GPEET.* These four genes are present in two copies per diploid genome (Ferguson, 1997; Roditi *et al.*, 1998; Vassella *et al.*, 2001). Transcription is polycistronic and can occur simultaneously from two or more expression sites (Acosta-Serrano *et al.*, 1999). EP-1 and EP-3 are post-translationally modified to contain N-linked, homogeneous $Man_5GlcNAc_2$ oligosaccharides in the N-terminal domain and GPEET procyclin is modified by addition of phosphates to six of seven threonine residues. All procyclins are anchored to the membrane by a GPI linkage with an unusual lipid structure (Ferguson, 1997; Treumann *et al.*, 1997; Mehlert *et al.*, 1998; Acosta-Serrano *et al.*, 2000; Acosta-Serrano *et al.*, 2001; Vassella *et al.*, 2001). The GPI anchors are highly glycosylated and contain large, heterogeneous side chains of branched poly-N-

acetyllactosamine repeats terminated with sialic acid residues that are transferred from a membrane-associated trans-sialidase **(Figure 5)**. The glycosylated GPI anchors of procyclins are partially responsible for the anomalous migration of these proteins on SDS-polyacrylamide gels (Ferguson, 1997; Roditi *et al.*, 1998; Acosta-Serrano *et al.*, 2001). The EP procyclins migrate as a diffuse band from 30-40 kDa and the phosphorylated GPEET procyclins migrate at 22-32 kDa (Richardson, 1988; Butikofer *et al.*, 1997; Roditi *et al.*, 1998).

iii. Functions of procyclins

Analysis by nuclear magnetic resonance, molecular modeling and immunoelectron microscopy indicates that the procyclin anchors form a glycocalyx immediately adjacent to the membrane, above which extend rod-like structures of the procyclin polypeptides (Roditi, 1990; Ferguson, 1997; Mehlert *et al.*, 1998). Although it has been 18 years since their discovery there is yet to be a well defined function for procyclins. It has been reported that the N-terminal domains of all procyclins are quantitatively removed by proteolysis within the midgut, however the C-terminal EP and GPEET repeats remain intact (Acosta-Serrano *et al.*, 2001; Vassella *et al.*, 2001; Butikofer *et al.*, 2002; Aksoy *et al.*, 2003). It has also been shown that EP null mutants or those with no surface procyclins (both EP and GPEET procyclins removed) show greatly reduced infectivity in the fly (Vassella *et al.*, 2003). Both of these observations suggest a protective role for procyclins. The dense glycocalyx surrounding the parasites may contribute to protection from digestive enzymes (proteases) within the midgut upon

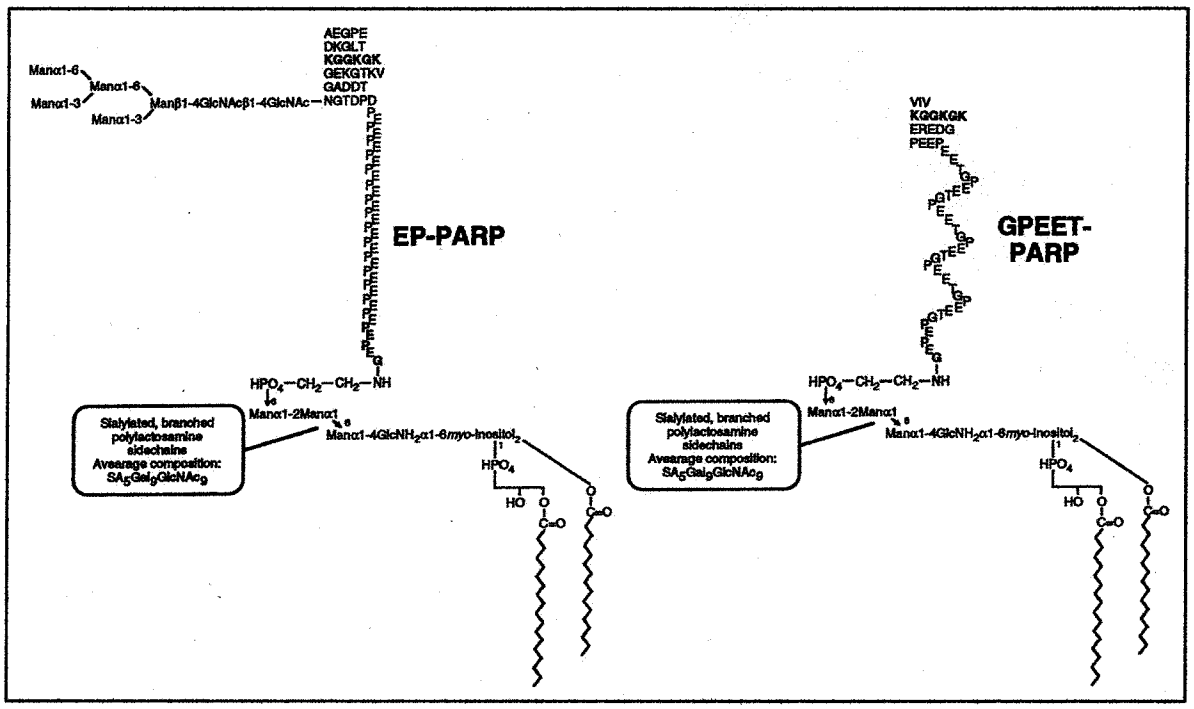

Figure 5. Rod-like structures of *T. brucei* EP and GPEET PARPs (procyclic acidic repetitive repeats) or procyclins. Both contain GPI anchors with an unusual lipid structure and large carbohydrate side chains made of polylactosamine repeats. (Mehlert *et al.*, 1998)

initial infection (Mehlert *et al.*, 1998; Roditi *et al.*, 1998; Acosta-Serrano *et al.*, 1999; Ferguson, 1999; Acosta-Serrano *et al.*, 2001; Pearson, 2001; Vassella *et al.*, 2001; Butikofer *et al.*, 2002; Vassella *et al.*, 2003). It has also been suggested that procyclins act as a target for two immune activities identified in tsetse flies: production of a trypanocidal factor which kills procyclic forms and production of another factor that stimulates further differentiation and migration of the parasites to the mouth parts (Stebeck *et al.*, 1994; Roditi *et al.*, 1998; Acosta-Serrano *et al.*, 1999; Mookherjee *et al.*, 2001). These activities can be modified by feeding infected flies sugars that inhibit midgut lectin activity, which increases infectivity rates of the trypanosomes (Maudlin, 1987). Therefore the trypanocidal factors could be lectins themselves which bind to N-

linked carbohydrates on procyclins (Roditi *et al.*, 1998; Acosta-Serrano *et al.*, 1999; Butikofer *et al.*, 2002). Inhibition of midgut lectin activity has also been shown to inhibit trypanosome development and maturation indicating the importance of tsetse-trypanosome interactions (Maudlin, 1988). It is a frustrating reality that no lectins have been biochemically purified or functionally characterized since their existence was postulated.

iv. Regulation of procyclin expression

Viable, transformed, procyclic trypanosomes become apparent in the tsetse blood meal in the tsetse midgut within the first two to four hours and spread throughout the lumen of the anterior midgut where they divide exponentially for 24-72 hours (Roditi *et al.*, 1998; Aksoy *et al.*, 2003). As trypanosomes develop in the tsetse vector they express different isoforms of procyclins in a regulated manner that has been characterized. Upon initial infection, all procyclins are synthesized at the onset of differentiation (Acosta-Serrano *et al.*, 2001; Vassella *et al.*, 2001). By day three post infection the trypanosomes are either completely eliminated from some infected flies or proceed to invade the ectoperitrophic matrix. At this stage the surviving parasites express only GPEET procyclins that have been cleaved near the N-terminus and shortened by 11 residues. By days 7-9 GPEET procyclins are replaced by the N-glycosylated forms of EP procyclins, EP-1 and EP-3. The EP procyclins also display a cleavage near the junction of the N-terminus and the extensive EP repeats (Roditi *et al.*, 1998; Acosta-Serrano *et al.*, 2001; Vassella *et al.*, 2001). This modulated expression of procyclins suggests that the different isoforms display various functional roles, as yet undefined. Trypanosomes

continue to differentiate as they migrate from the midgut to the salivary glands where expression of a specific subset of VSG genes is activated for transmission to a new mammalian host (Ruepp *et al.*, 1997; Roditi *et al.*, 2002).

1.3 Glycoconjugates of other procyclic stage trypanosomes

It should be noted that other trypanosome species have the equivalent of procyclins, suggesting their importance in parasite development and tropism in the tsetse fly. Using mAbs derived against the procyclic form of *Trypanosoma congolense,* a new surface glycoprotein was identified and characterized (Beecroft, 1993). The mAbs raised against this surface molecule only bound to the procyclic stage of *T. congolense* and *T. simiae*. The protein was characterized as an acidic, glycoconjugate rich in carbohydrate, not dissimilar to procyclins except there was no sequence similarity between the two (Beecroft, 1993). The protein is now known as GARP (glutamic acid-alanine rich protein) in *T. congolense* (Mookherjee *et al.*, 2002). Fluorescence-activated cell sorting showed that the kinetics of GARP expression during transformation from BSF to PCF was similar to that of procyclins expressed by *T. brucei sspp. T. simiae* does not contain the gene for GARP, yet mAbs specific for procyclic parasites recognized carbohydrate epitopes on the surface of both *T. simiae* and *T. congolense* (Mookherjee *et al.*, 2002). Thus *T. simiae* also expresses a carbohydrate rich surface glycoconjugate that shares a common carbohydrate epitope with GARP, albeit displayed on a different polypeptide molecule (Mookherjee *et al.*, 2002). *T. congolense* and *T. simiae* show different developmental cycles in the insect vector when compared to *T brucei sspp.* Therefore the identification of procyclin-like molecules on the surface of other procyclic stage

trypanosomes suggests that these surface glycoconjugates play critical roles in trypanosome-tsetse interactions perhaps by influencing parasite tropism in the vector (Beecroft, 1993; Mookherjee *et al.*, 2002).

1.4 Non-variant surface molecules

After the discovery of antigenic variation it quickly became apparent that a vaccine based on the VSG antigen was not likely to be feasible. Therefore, the focus for vaccine development and drug design has recently turned to invariant surface molecules that may be located underneath the major surface coat proteins or within the flagellar pocket (Pays *et al.*, 1998). Parasites depend upon their hosts for nutrients, therefore the receptors and transporters required for food uptake would be good target candidates for chemo- or immunotherapy because they are also unlikely to undergo antigenic variation (Ziegelbauer *et al.*, 1992; Nolan *et al.*, 1997; Borst *et al.*, 1998; Pays *et al.*, 1998). These molecules are important because of the functions they may perform such as: interactions with their mammalian hosts, differentiation, and possibly inter-trypanosome interactions (Nolan *et al.*, 1997; Pays *et al.*, 1998).

Isolating and characterizing these non-variant surface antigens has proven very challenging (Ziegelbauer *et al.*, 1992; Ziegelbauer *et al.*, 1992; Nolan *et al.*, 1997; Borst *et al.*, 1998; Pays *et al.*, 1998). This is mainly due to the nature of the major surface glycoproteins; VSG on the BSF and procyclins on the insect midgut forms. These glycoproteins form macromolecular diffusion barriers covering the entire expanse of the parasite surface including the flagellum. Consequently, these major surface antigens shield the underlying non-variant proteins from components of both the innate and

humoral immune responses. The isolation of invariant surface proteins is also hindered by their very low abundance, relative to the major surface glycoproteins; 100- 10,000-fold less (Ziegelbauer *et al.*, 1992; Ziegelbauer *et al.*, 1992; Nolan *et al.*, 1997; Borst *et al.*, 1998; Pays *et al.*, 1998). Surface labeling experiments to identify membrane embedded surface proteins have also proved difficult, or useless, because they cannot penetrate the protective coat and reach the membrane surface. Over the years the number of invariant surface molecules discovered has slowly increased, but there is still only a few known today. These include: a transferrin receptor, LDL and HDL receptors, ion ATPase pumps, glucose transporters, a metalloprotease and a number of invariant surface glycoproteins (ISGs) with unknown functions (Ziegelbauer *et al.*, 1992; Ziegelbauer *et al.*, 1992; Nolan *et al.*, 1997; Barrett *et al.*, 1998; Borst *et al.*, 1998; Carrington *et al.*, 1998; Pays *et al.*, 1998; De Koning, 2001; De Koning, 2001; Green *et al.*, 2003; LaCount, 2003; Stiles *et al.*, 2003).

i. The transferrin receptor

Iron is an essential host-derived growth factor for BSF trypanosomes, which suggested the presence of a specific transferrin receptor. In 1987 Coppens and colleagues published the first evidence to support the presence of a transferrin receptor (Tf-R) on *T. brucei* (Borst *et al.*, 1998). In 1991 Schell and colleagues discovered that the product of one of the VSG expression site associated genes, *esag 6,* was a component of the purified Tf-R complex. In 1993 the collaboration between the Overath and Borst labs lead to the demonstration that purified Tf-R contained two proteins in equimolar amounts, ESAG 6 and ESAG 7, forming a heterodimer. These were the first proteins encoded by *esag*s

(Expression Site Associated Genes) to be functionally identified. The pESAG 6 contained a C-terminal hydrophobic extension where the GPI anchor is added, and by which the protein is attached to the membrane. More recently it has been observed that the Tf-R is evolutionarily related to VSGs because of structural similarities and sequence homology. However, unlike VSGs, it is not distributed over the entire surface but is found only in the FP and in small numbers, estimated at 3000 receptors per cell (Borst *et al.*, 1998; Pays *et al.*, 1998).

ii. Adenylate cyclase

In addition to *esag 6* and *esag 7*, another gene contained within the polycistronic transcription unit of the *VSG* gene, termed *esag 4,* was found to encode a surface protein (Borst *et al.*, 1998; Pays *et al.*, 1998). The gene encoded an adenylate cyclase which belongs to a larger family of genes, some of which also encode adenylate cyclases. These cyclases all share common receptor-like structures: a large, variable extracellular domain, a single transmembrane domain and a highly conserved intracellular catalytic domain predicted to be located directly underneath the plasma membrane (Borst *et al.*, 1998; Pays *et al.*, 1998). *Esag 4* encodes a Ca^{2+}-regulated adenylate cyclase expressed by BSF on the surface and in the FP (Borst *et al.*, 1998; Pays *et al.*, 1998). This cyclase probably responds to extracellular stress, perhaps during differentiation from BSF to PCF in the insect midgut. Adenylate cyclase syntheses cyclic AMP from ATP, a second messenger that links extracellular signals with intracellular events (Kelly, 2004). Cyclic AMP can bind to and activate a number of intracellular receptors including protein

kinases, key regulators of signaling pathways, that trigger cascades of downstream events leading to cellular response (Kelly, 2004).

iii. Lipid receptors

Trypanosomes utilize lipids extensively, especially for the generation of GPI anchors for surface proteins. Given this requirement it is interesting that trypanosomes remain lipid auxotrophs and therefore require lipoproteins and other serum components in order to synthesize the fatty acids and cholesterol they require (De Koning, 2001; Green *et al.*, 2003). Low density lipoprotein (LDL) and high density lipoprotein (HDL) deliver lipids in the form of cholesterol, cholesterol esters and phospholipids to the parasite. Both BSF and PCF *T. b. brucei* specifically take up both LDL and HDL via receptor-mediated endocytosis through the flagellar pocket. There is evidence to support the presence of a LDL specific receptor in the flagellar pocket although only a putative receptor has been purified and the gene has yet to be cloned (Borst *et al.*, 1998; Pays *et al.*, 1998; Green *et al.*, 2003). HDL is also taken up by receptor mediated endocytosis but there has been no molecular characterization of a HDL receptor in BSF (Green *et al.*, 2003). Human serum contains the HDL-related factor termed trypanosome lytic factor (TLF) that is responsible for the host range restriction of the *T. b. brucei* subspecies (Pays *et al.*, 1998; Green *et al.*, 2003). The lysis of susceptible strains requires the uptake of TLF in the flagellar pocket and delivery to lysosomes where lysosomal disruption leads to autodigestion of the cell (Pays *et al.*, 1998). It seems that the mechanism of resistance is not due to the lack of receptor, but to the failure to effectively process receptor bound TLF (Pays *et al.*, 1998). It has been recently shown by Green (2003) that TLF competes

for binding with LDL and HDL for a selective lipoprotein scavenger receptor. This scavenger selectively binds the lipid component of LDL and HDL over the protein component and it is the competition between TLF, LDL, and HDL that attenuates trypanosome TLF-mediated lysis (Green *et al.*, 2003).

iv. Glucose transporters

Glucose is a main carbon source used by all trypanosome species, and all have a specific plasma membrane transporter to facilitate its uptake. It appears that the transporters have evolved to best suit the needs of the parasite depending on which life cycle stage it is in and therefore the different extracellular environments it encounters. *T. b. brucei* contains two different glucose transporter isoforms, THT1 and THT2 (trypanosome hexose transporter). These two proteins are estimated to be ~53 kDa, contain twelve membrane spanning regions, a single N-glycosylation site and a cysteine rich hydrophilic loop and are thought to be expressed on the cell surface. These two isoforms are 80% identical and differ mainly in their carboxy terminal domains. Using northern blot analysis it was found that BSF express 40 fold higher levels of THT1 with respect to THT2, while THT2 is more abundant in PCF. The expression of two different glucose transporter isoforms in *T. b. brucei* is explained by the different kinetic properties of transport in BSF compared to PCF. BSF utilize glucose as their main carbon source where as PCF switch to amino acids, namely proline, and glucose becomes a secondary source. Therefore BSF possess a low affinity, high capacity glucose uptake system (THT1) to exploit the glucose rich environment in mammalian blood. In contrast the

PCF possesses a high affinity, low capacity system (THT2) suitable for the insect midgut where glucose is scarce (Barrett *et al.*, 1998; Borst *et al.*, 1998; Pays *et al.*, 1998).

v. Membrane bound proteases

The first major surface protease (MSP) of a protozoan parasite was discovered in *Leishmania* species and was previously called GP63. GP63 is a cell-surface, zinc-dependent metalloprotease, which was later, found to contribute to parasite entry and survival in macrophages. Therefore, LaCount *et al.* (2003) were surprised to find that African trypanosomes also contain genes that encode homologues of the *Leishmania* MSPs, given their exclusive extracellular life cycle (LaCount, 2003). They thought it possible that MSPs expressed by African trypanosomes might function to provide the previously uncharacterized cell surface metalloprotease activity that can release ectopically expressed surface proteins. Three differentially expressed MSP genes were discovered, called *TbMSP-A, -B* and *–C*, although there may be more. The three proteins encoded by these genes range in size from 591 to 622 amino acids of which 33% of the amino acids are identically positioned within the polypeptide (LaCount, 2003). The MSPs differ most at their carboxy termini. All three are expressed by BSF trypanosomes but only *TbMSP-B* is expressed by the procyclic stage. Because TbMSP-B is expressed by both life cycle stages it was hypothesized that it may provide an essential function for the parasite. However, the use of RNA interference (RNAi) showed that TbMSP-B was not essential for growth of either life cycle stage (LaCount, 2003). During all stages the parasites are coated with surface proteins that constitute 5-10% of the total cellular protein. This membrane coat is continually being turned over, either as the VSGs switch

to avoid immune elimination from the mammal, or the procyclins switch as the parasite differentiates and migrates in the vector. This places a huge burden on the parasite mechanisms for protein secretion and surface protein turnover (LaCount, 2003). A model system was previously derived in which VSG is constitutively expressed by PCF, where it is transported to the surface, and then released in a truncated form by an endoprotease. This endoprotease can be inhibited by various metalloprotease inhibitors; therefore with the discovery of MSPs expressed by *T. brucei* it became possible that TbMSP-B could be responsible for this proteolysis. RNAi was used to knockdown TbMSP-B expression in PCF cells expressing VSG and this inhibited release of the truncated VSG into the medium. Therefore, they found TbMSP-B responsible for the previously uncharacterized metalloprotease activity that releases VSG from procyclic cells (LaCount, 2003). However, TbMSP-B might also be responsible for the endoproteolytic activity in combination with GPI-specific phospholipase C (GPI-PLC) that cleaves off VSG during differentiation (LaCount, 2003).

GPI-PLC cleaves the GPI anchor of membrane bound proteins, releasing them into the extracellular fluid. In trypanosomes, VSG is the main substrate for GPI-PLC due to its great abundance. However, there are other GPI-linked proteins that act as substrates. It is unknown how the GPI-PLC is attached to the membrane as there are no conserved amino acid sequences within the polypeptide that suggest membrane association domains or anchor attachment signal sequences. This contrasts with the fact that GPI-PLC requires detergent for solubilization. The GPI-PLC is responsible for GPI-anchor hydrolysis on the VSG, releasing dimyristyl glycerol and converting the hydrophobic membrane form of VSG to a water soluble form. In this way *T. b. brucei*

sheds its VSG coat, which it must do during three different phases in the life cycle: 1) A few days after entry into the mammalian host when metacyclic VSG is exchanged for BSF VSG. 2) When an individual trypanosome undergoes antigenic variation. 3) During differentiation from BSF to PCF in the tsetse midgut where VSG is replaced by procyclins. It is hypothesized that GPI-PLC is involved in these three events when VSG is released from the cell (Carrington *et al.*, 1998). With the recent evidence to support a role for MSPs in VSG release, it may prove to be a combination of both GPI-PLC and MSP activity that removes VSG from the cell surface.

vi. Ion pumps

There is extensive evidence to support the presence of proton and calcium pumps in trypanosomes. However, very little is known about the role of sodium (Na^{2+}) and potassium (K^{+}) pumps in these parasites. In eukaryotic cells K^{+} is the most abundant cation present inside the cell, while Na^{2+} levels are kept relatively low (Stiles *et al.*, 2003). These pumps transport K^{+} and Na^{2+} ions across the plasma membrane and are responsible for maintaining the plasma-membrane potential that drives secondary transport of nutrients. Stiles *et al.* (2003) recently identified a surface membrane K^{+}/Na^{2+} P-type ATPase, called TBCA1, in *T. b. brucei.* This protein is 150 kilodaltons (kDa) and using immunolocalization was found to be localized to the sub-pellicular region, the flagellar pocket and the flagellum in both BSF and PCF (Stiles *et al.*, 2003).

vii. Purine transporters

BSF trypanosomes need to salvage purines from the blood stream and do so by transporters thought to be located in the plasma membrane. These include the purine ribonucleoside transporters P1 and P2 and the purine nucleoside transporters H1, H2 and H3 (Barrett, 1999; De Koning, 2001). Uptake of adenosine, inosine and guanosine is mediated by the P1 transporter, adenine and adenosine by the P2 transporter and adenine, guanine and hypoxanthine by the H1, H2 and H3 transporters (Barrett, 1999; De Koning, 2001). These transporters also act as carriers for trypanocidal drugs and it is hypothesized that loss of one or more of these transporters leads to drug resistance (Barrett, 1999; De Koning, 2001; De Koning, 2001; Denise *et al.*, 2001). In all examples of cross-resistance between diamidines and arsenicals studied to date, the P2 transporter has been altered in some way that leads to decreased drug uptake and consequently to drug resistance. Even though they have different intracellular targets, diamidines (ie. pentamidine) and melaminiophenyl arsenicals (ie. melarsoprol) obviously share a common system for uptake (although this may not be an exclusive mode of uptake) using P2-based transport (Barrett, 1999; De Koning, 2001; De Koning, 2001; Denise *et al.*, 2001). More recently, other transporters for pentamidine have been reported including a high affinity pentamidine transporter (HAPT1) and a low affinity pentamidine transporter (LAPT1) (Barrett, 1999; De Koning, 2001).

viii. Invariant surface glycoproteins (ISGs)

In 1992 Ziegelbauer *et al.* (1992) isolated three novel glycoproteins, subsequently called invariant surface glycoproteins (ISGs): ISG60, ISG65 and ISG75, the numbers

corresponding to their apparent molecular weights (Ziegelbauer *et al.*, 1992; Ziegelbauer *et al.*, 1992). These proteins were only isolated from BSF *T. b. brucei* and could not be found in the procyclic stage (Ziegelbauer *et al.*, 1992; Ziegelbauer *et al.*, 1992). In 1997 Nolan *et al.* published the discovery of another ISG, termed ISG100, that is also expressed strictly in BSF (Nolan *et al.*, 1997). The protein encoded by the *Isg100* gene is found associated with the flagellar pocket and components of the endo/exocytosis pathways, such as intracellular vesicles located in close proximity to the pocket. This protein, like the other ISGs, was highly glycosylated but unique due to its very large, internal serine rich, 17 amino acid repeat (Nolan *et al.*, 1997). The genes and their encoded ISG proteins show no sequence similarity to any other known genes or proteins and therefore their functions remain elusive (Ziegelbauer *et al.*, 1992; Ziegelbauer *et al.*, 1992; Borst *et al.*, 1998; Pays *et al.*, 1998).

1.5 Critical roles of GPI-anchors for *T. brucei*

All life cycle stages of trypanosomes use GPI anchors as the predominant method for attaching proteins to their plasma membranes (Lillico *et al.*, 2003). In the mammalian host, trypanosomes express at least the following proteins: a transferrin heterodimeric receptor, an alanine-rich protein with unknown function and VSG (Lillico *et al.*, 2003). GPI anchors are synthesized in the endoplasmic reticulum by sequential addition of sugars and ethanolamine phosphates onto phosphatidylinositol (Ferguson, 1999; Lillico *et al.*, 2003). Precursor proteins destined for addition of a GPI anchor contain an endoplasmic reticulum (ER)-directing signal sequence which is cleaved upon translocation into the ER and a C-terminal domain comprising a GPI anchor addition

signal sequence (Ferguson, 1999; Lillico *et al.*, 2003). The C-terminal GPI addition signal sequence is recognized by a transamidase complex that proteolytically cleaves the protein and adds a precursor-GPI anchor to the terminal ethanolamine phosphate **(Figure 6)** (Lillico *et al.*, 2003).

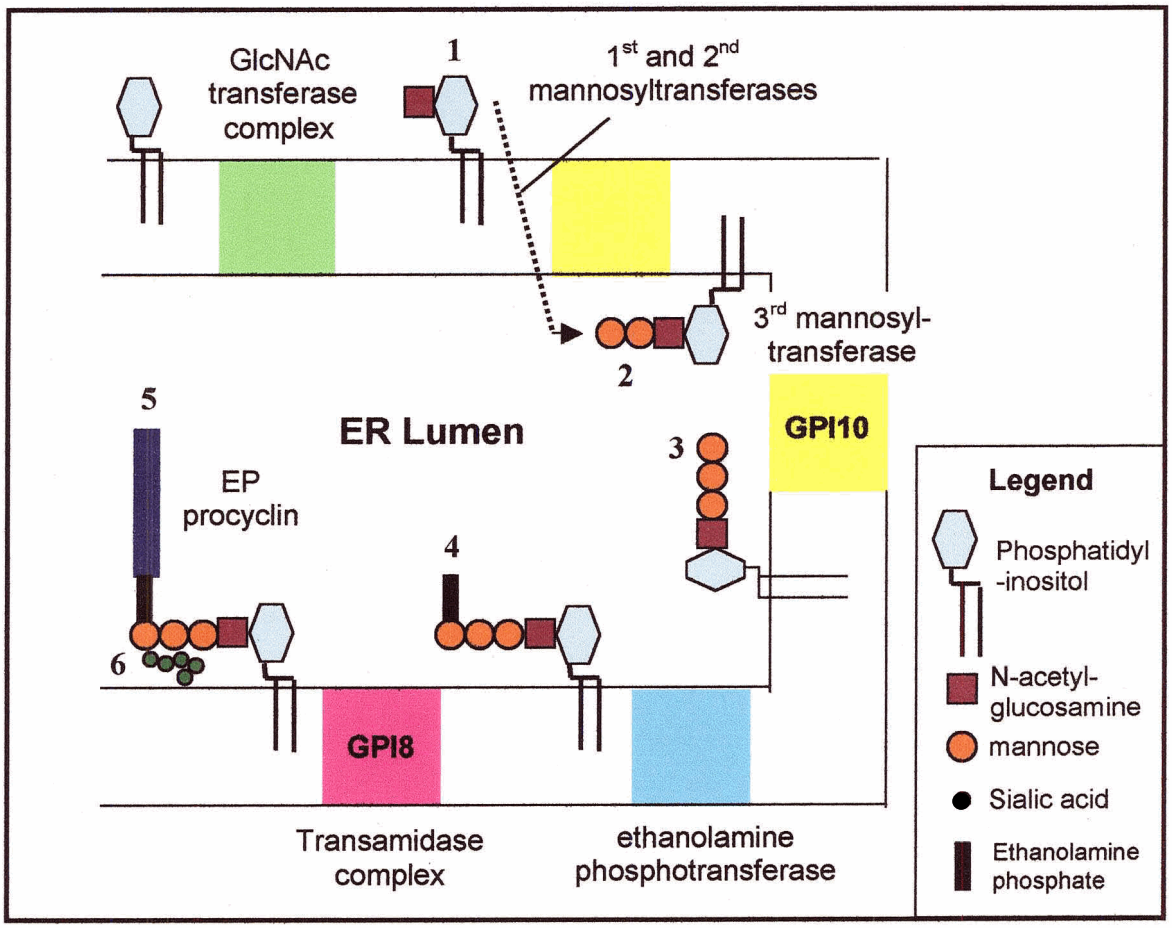

Figure 6. Simplified diagram of GPI biosynthesis in *T. brucei* procyclic forms. All of the enzymes are present in the ER membrane and GPI synthesis and addition to a protein occurs here. 1) The N-acetylglucosamine (GlcNAc) transferase complex adds a GlcNAc molecule to the phosphatidylinositol. 2) The first and second mannose moieties are added by unknown mannosyltransferases. 3) The third mannosyltransferase is GPI10, a trypanosome homologue of the mammalian PIG-B protein. 4) An unknown ethanolamine phosphotransferase adds the ethanolamine phosphate group in preparation for 5) addition of the protein which is transferred by a transamidase complex comprised of GPI8 and other unknown proteins. 6) Carbohydrate remodeling occurs as sialic acid moieties are added to the second mannose group. (Ferguson, 1999)

The *GPI10* gene of *T. b. brucei* is homologous to the human *PIG-B* gene that is involved in the third mannosyltransferase reaction in GPI anchor biosynthesis. Disruption of this gene causes complete loss of GPI anchors and results in the lack of GPI attachment to relevant proteins. Therefore, *GPI10* gene knock out trypanosomes should not be able to express any GPI-linked proteins on their surface. Nagamune *et al.* (2000) attempted to knock out the *GPI10* gene in BSF and PCF *T. b. brucei.* In BSF, they were unsuccessful and it was concluded that GPI10 is essential for viability of the BSF stage of trypanosomes. This could be due to the lack of VSGs expressed on the cell surface, or other essential proteins that would also not be present. For example, the transferrin receptor which has previously been described as an essential receptor for BSF growth would be necessary for survival. In contrast, when *GPI10* gene expression was disrupted in PCF, the knock outs were viable. However, if grown in tissue culture treated flasks, these knock out mutants adhered to the flask surface, elongated and died within several days. This indicated that these mutants might have an unusually sticky coat maybe in part due to the lack of procyclins. The GPI knock outs were able to grow and divide if grown in non-tissue culture treated flasks, to allow growth of non-adherent cells. Even under optimal growth conditions their growth rate was only half that of wild type cells *in vitro*.

The GPI8 cysteine protease is an essential component of the protein transamidase required for GPI anchor biosynthesis (Lillico *et al.*, 2003). Lillico *et al.* (2003) discovered that GPI8 is a soluble ER protein. Generation of *gpi8* knock out BSFs was unsuccessful, however, *gpi8* knock out PCFs were generated. They found that PCFs containing a *gpi8* knock out resulted in parasites lacking proteins on their surface, but

did result in the accumulation of precursor GPIs (Lillico *et al.*, 2003). Staining of proteins in a detergent, CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) extracted membrane fraction, showed that proteins were expressed by wild type cells that were not expressed by the *GPI8* knock out. There also seemed to be a subset of proteins expressed at much higher levels in the mutants when compared to wild type. This was also apparent when these cells were treated with the lectin Concavalin A. The GPI8 knock out parasites subsequently died, similar to wild type PCF. Therefore, there were other mannose-bearing structures on the cell surface, other than the usual ligand for Concavalin A, the N-linked glycans of EP-procyclins (Lillico *et al.*, 2003).

Lillico *et al.* (2003) predicted that their observations of the *GPI8* knock out would be similar to those found by Nagamune *et al.* (2000) for the *GPI10* knock out (Lillico *et al.*, 2003). The only common finding was the requirement of both GPI10 and GPI8 for the viability of BSF trypanosomes, meaning that GPI biosynthesis is essential for this life cycle stage. For both knock outs the PCF were viable. However the GPI10 mutants had to be grown in non-tissue culture treated flasks as they adhered to the plastic, unlike the GPI8 mutants (Nagamune *et al.*, 2000; Lillico *et al.*, 2003). The *GPII0* knock outs were also able to establish infection within the tsetse midgut, even though their cell cycle was longer when compared to wild type parasites (Nagamune *et al.*, 2000). The *GPI8* knock outs, with a comparable cell cycle length to wild type were unable to establish midgut infections. At the biochemical level, the GPI8 null mutants accumulated complete preformed GPI substrates of the transamidase complex (PP1) and appeared to express part of this accumulated pool of substrates on their surface (Lillico *et al.*, 2003). The *GPI10* knock outs were unable to express free GPIs which may explain their requirement

for non-adherent flasks. However, they expressed different mannolipids on their surface when compared to wild type PCF (Nagamune *et al.*, 2000; Lillico *et al.*, 2003).

1.6 Hypothesis

It is apparent that the major surface molecules of the procyclic form of *T. b. brucei*, the procyclins, complicate and hinder the identification and characterization of other molecules present on the parasite surface. My hypothesis is that by using procyclin knock out mutants of procyclic stage trypanosomes, it will be possible to identify underlying, less abundant surface molecules using a combination of immunological and biochemical techniques.

1.7 Research strategy

From the previous review of surface molecules expressed by *T. b. brucei* it is apparent that not only VSG and procyclins are important in the parasite life cycle. Less abundant, non variant surface proteins also play critical roles in parasite growth, differentiation and interactions with the mammalian host and insect vector. It would be ideal to isolate and characterize invariant molecules expressed on the surface of wild type BSF trypanosomes. However, the tightly packed coat of VSG greatly hinders the biochemical identification of underlying molecules. Creating a VSG knock out in BSF trypanosomes is impossible due to the fundamental role of VSGs in parasite viability and the vast number of VSG genes in the genome. However, it has been possible to create PCF knock out mutants that do not express the major procyclins or other proteins.

Therefore, to identify surface-exposed membrane molecules on *T. b. brucei* PCF is a desirable goal.

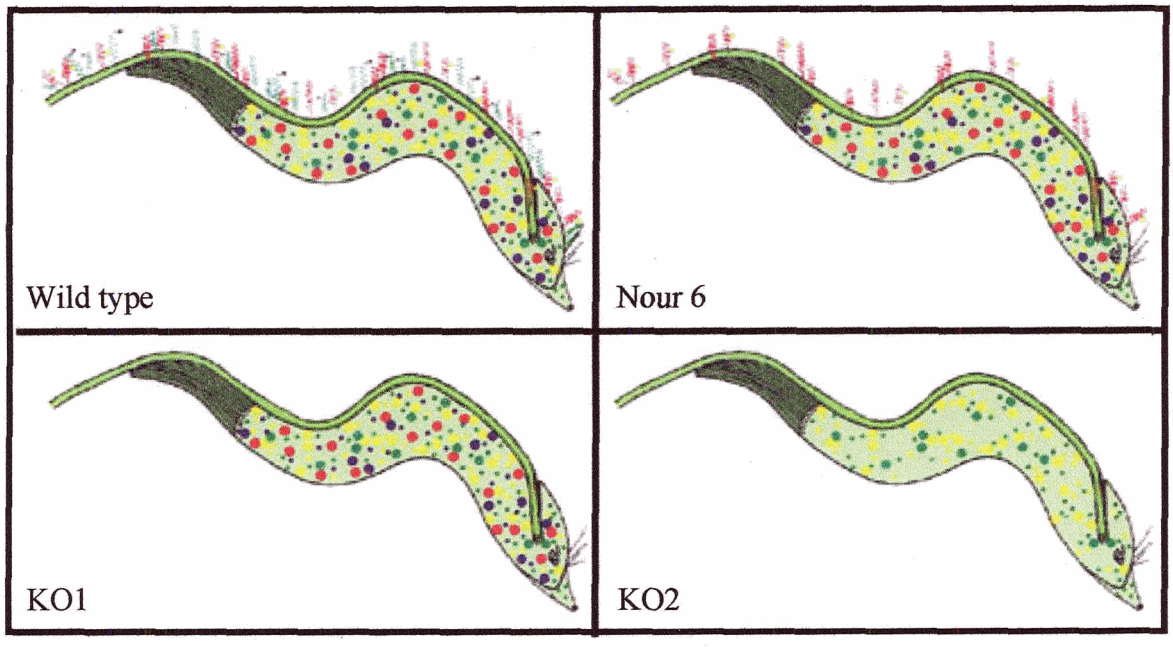

Figure 7. Schematic representation of the four *T. b. brucei* PCFs used in this research. Wild type PCFs express surface EP and GPEET procyclins. Nour 6 PCFs contains a knock out of the three genes encoding EP procyclins and expresses surface GPEET procyclins. KO1 PCFs contain knock out mutation of all procyclin genes and are therefore naked trypanosomes. KO2 PCFs contain a *GPI10* knock out and do not express procyclins or other GPI-anchored proteins on their surface. EP procyclins; green surface molecules, GPEET procyclins; red surface molecules, GPI-anchored surface proteins; red and blue circles. This figure is modified from the 'Spiny Norman' model (Pearson, 2001).

My strategy included using procyclin knock out mutants for identification of non-procyclin molecules on the surface of *T. b. brucei* PCF trypanosomes. These mutants included Nour 6 (EP procyclin knock out), KO1 (EP and GPEET procyclin knock out), and KO2 (GPI10 knock out that does not express any GPI-anchored surface proteins) **(Figure 7)**. My first approach was to characterize the protein profiles of wild type PCF and three surface knock out PCF mutants. My second approach was to generate

monoclonal antibodies to the non-procyclin expressing cell surface, of knock out PCF mutants, and to identify the relevant antigens by protein mass spectrometry. Finally, identification of novel cell surface proteins on trypanosome cell surface mutants was approached by surface biotinylation of three different surface mutants of PCF trypanosomes and identification of labeled molecules by protein mass spectrometry.

2. Materials and Methods

2.1 Trypanosomes

Procyclic culture forms (PCF) of *T. b. brucei* 427.01 wild type (Cross, 1973), and the derivative clones Nour 6C (an EP procyclin knock out mutant; (Ruepp *et al.*, 1997) KO1 (an EP/GPEET procyclin knock out; (Vassella *et al.*, 2003)) were cultured at 27 °C in modified minimal essential medium containing 10% heat-inactivated fetal bovine serum as previously described (Fish, 1989). The GPI knock out mutant TbGPI10 (mutant of *T. b. brucei* 427.221a PCF; (Nagamune *et al.*, 2000)), was cultured at 27 °C in SDM-79 medium containing 10% heat-inactivated fetal bovine serum (Brun, 1979). The GPI mutants were grown in non-tissue culture treated flasks (Becton Dickinson Falcon, NJ, USA). Blood-stream form (BSF) 427.01 Clone 1 trypanosomes were grown in Baltz medium (Baltz, 1985). When first thawed, BSF trypanosomes were serially diluted and grown in 96 well and 24 well tissue culture plates. After 18 – 22 hours the cells were fed with warmed Baltz medium. Once the cells reached log phase and an area of turbid culture could be seen in the wells, they were transferred from 96 well to 24 well plates with an equal volume of fresh medium. Cells were finally transferred to 50 mL tissue culture flasks when showing signs of logarithmic growth.

Cells were counted using a hemacytometer before every experiment to determine the number of cells per mL of culture. The volume required for the number of cells needed for the experiment was calculated and five times that volume was extracted from the tissue culture flask and centrifuged at 150 X g. The cells were washed twice with PBS (pH 7.4), resuspended in 1 mL PBS and recounted using the hemacytometer. The

volume needed to provide the required number of cells was measured and transferred into a new microcentrifuge tube. The cells were centrifuged and resuspended in the appropriate solution for use in the rest of the experiment.

2.2 Cryopreservation of Trypanosomes

Trypanosomes were preserved by freezing in liquid nitrogen. For each trypanosome culture, 10 mL of freezing medium (50% culture medium, 20% glycerol, 20% FBS) were filter sterilized using a 0.2 μm syringe filter. The medium used was either PCF medium for wild type, Nour 6 and EP/GPEET KO1 PCF trypanosomes, SDM-79 for the GPI knock out PCF trypanosomes and Baltz medium for BSF trypanosomes. Cells from healthy, rapidly dividing cultures (50 mL) were centrifuged at 1000 X g for 6 min at room temperature. The supernatants were removed and the cells were gently resuspended in 6 mL of freezing medium. The cells were then dispensed (1 mL each) into labeled cryovials and placed into a freezing head of a liquid nitrogen tank to be left over night to slowly cool before sinking the following day. Trypanosome cultures were maintained until one vial of frozen stock was thawed to ensure viability of the preserved cells.

2.3 One dimensional polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970) using a Mini-protean II apparatus (Bio-Rad Laboratories, Hercules, CA, USA.). Unlabeled and biotin labeled proteins were separated using a 3% stacking gel run at 50 mA for 30 minutes and a 10% separating gel

run at 110 mA for 1.5 hours. Separated proteins were stained with either colloidal Coomassie Brilliant Blue G-250 or silver nitrate (see below), or were transferred to polyvinylidene fluoride (PVDF) membrane for immunoblot analysis. Unstained molecular mass markers (10-200 kDa; catalog No. SM0661; Fermentas Life Sciences Inc., Hanover, MD.) were run on all gels to be stained. Pre-stained molecular mass markers 10-160 kDa (catalog No. SM0671 Fermentas Life Sciences) were run on gels to detect the efficiency of electrophoretic transfer onto PVDF membranes and to serve as molecular mass standards.

2.4 Two dimensional polyacrylamide gel electrophoresis

High-resolution two-dimensional SDS-PAGE was performed using the ISO-DALT multiple 2D gel system (Anderson, 1978a; Anderson, 1978b) as previously described (Pearson, 1987). Typically 2 X 10^7 trypanosomes were solubilized in 25 µL of urea mix (9 M urea, 4 % (v/v) NP40, 2 % (v/v) ampholytes, 2 % (v/v) mercaptoethanol, pH 9.5). Ampholines used for first-dimension gels were Pharmalyte pH 3-10 (Amersham Pharmacia, Uppsala, Sweden) and samples were run for 14, 400 volt-hours. Second dimension separations were performed using gradient SDS-PAGE slab gels (10-16.5%) run at 1.0 A for 4-5 hours. Gels were either stained with colloidal Coomassie Brilliant Blue G-250 or used for transfer to PVDF membranes for immunoblotting.

2.5 Colloidal Coomassie Brilliant Blue G-250 staining of proteins in gels

1D and 2D gels were incubated in fixative (50 % v/v ethanol, 3 % v/v ortho phosphoric acid) overnight, washed 3 X 30 min in distilled water and allowed to

equilibrate in Neuhoff's solution (16 % w/v ammonium sulphate, 25 % v/v methanol, 5 % v/v ortho phosphoric acid; (Neuhoff, 1988) for one hour. All steps were performed at room temperature with gentle agitation. One gram of Coomassie Brilliant Blue G-250 (CBB, SERVA Blue G, research grade; SERVA Electrophoresis, Heidelberg, DRG) was then added into the Neuhoff's solution and staining continued for two to three days. A light box was used to observe the stained gels and after dark protein spots became apparent the gels were either scanned and protein spots cored, or the intact gels were transferred into a 20 % (w/v) ammonium sulphate solution for storage at 4 °C (Neuhoff, 1988). Digital images of stained gels were obtained by scanning at 300 dpi using a colour scanner (UMAX Astra 3400, Fremont, CA). The images were stored and manipulated (brightness/contrast/crop/label) in TIFF format using Photoshop™ 5.5 graphic software (Adobe Systems Inc., San Jose, CA).

2.6 Silver nitrate staining of proteins in gels

1D SDS-polyacrylamide gels were incubated in fixative (50% methanol/10% acetic acid) for 30 min at room temperature with gentle agitation. Gels were then incubated (10% methanol/10% acetic acid) for 30 min after microwaving (1.3 KW, 120 V, 65 Hz, A. C., maximum operating output; 720 W) on high for 1 min. The gels were then washed with distilled water for 5 min and then placed into reducing solution (1M DTT in water). The gels in reducing solution were microwaved on high for 1 min then washed vigorously 3 X 3 min in distilled water. Gels were then placed into silver stain (0.1% silver nitrate in water) (BDH Inc. Toronto, Canada) and incubated for 15 min with gentle agitation. The stained gels were washed with a steady stream of water for 1 min

and then placed into the developer solution (3% sodium carbonate/0.1% formaldehyde) for 1 to 3 min until the bands had appeared on the gel. To stop development, gels were removed from the developer and added to the stop solution (5% acetic acid) for 5 min with mixing. The gels were either dried or stored in 10% ethanol/7% glycerol at 4 °C.

2.7 Membrane preparation

To immunize more specifically with the antigens of interest (less abundant, invariant surface molecules of the KO1 EP/GPEET knockout mutants of *T. b. brucei*) trypanosome membranes were isolated from whole cells using a modified version of the protocol published by Nolan *et al.* (Nolan *et al.*, 1997). After washing, 10^9 trypanosomes were resuspended in 10 mL isotonic sucrose containing 1X protease inhibitor cocktail and then disrupted by nitrogen cavitation for 45 min at 2000 psi on ice. The protease inhibitor cocktail contained 500 μM AEBSF, HCl, 150 nM A protein, 1 μM E-64, 1 μM leupeptin, hemisulfate and 1 μM PMSF. The disrupted cells were centrifuged at 1000 x G for 4 min and the supernatant extracted. The pellet was resuspended, centrifuged again and the supernatants were combined and centrifuged at 60, 000 x G for 45 min. The high speed pellet, containing the disrupted membranes, was resuspended and carefully layered onto a discontinuous sucrose density gradient and subjected to ultracentrifugation at 60, 000 x G for 1.5 hours. Predicted bands in the sucrose density gradient were as follows: (1) plasma membrane at 1.22 g/ml, (2) microsomal/ER membrane at 1.175 g/ml and (3) flagellar pocket membrane at 1.11 g/ml (Nolan *et al.*, 1997). After ultracentrifugation, bands were apparent at 1.17 g/mL and 1.25 g/mL in the gradient. These bands were at densities very close to those predicted to contain membrane proteins and therefore were

extracted using a sterile glass pipet and dialyzed against PBS for 4 days to remove the sucrose. These samples were then concentrated and desalted using a Centricon centrifugal filter device (Millipore Corporation, Bedford, MA). The proteins in these fractions were analyzed by SDS-PAGE and quantified using a bicinchoninic acid assay (see below).

2.8 BCA protein assay of membrane fractions

The protein concentrations in the 1.17 g/mL and 1.25 g/mL fractions extracted from the sucrose density gradient were determined using a bicinchoninic acid (BCA) protein assay reagent kit following instructions provided by the manufacturer (PIERCE, Rockford, IL.). Bovine serum albumin standards were prepared as directed and the microplate procedure was followed in order to obtain the absorbance of each standard at 550 nm. A standard curve was prepared by plotting the absorbance for each standard versus its concentration in μg/mL. This standard curve was then used to determine the protein concentration of the dialyzed and concentrated 1.17 and 1.25 g/mL fractions extracted from the sucrose density gradient. The protein concentration of the fractions was required to determine the volume of sample that would be used for immunizations for derivation of monoclonal antibodies.

2.9 Immunizations

Four female BALB/c mice (10-14 weeks old) were selected for immunization. Two were immunized with live *T. b. brucei* KO1 EP/GPEET knock out mutants, while the other two received plasma membrane proteins extracted from the KO1 mutant

trypanosomes. Immunizations were performed with live trypanosomes (5 X 10^7 cells in 100 μL PBS injected intraperitoneally). The second pair of mice were injected intraperitoneally with plasma membrane preparations (50 μL containing approximately 550 μg of plasma membrane proteins from both the 1.17 g/mL and 1.25 g/mL fractions). This membrane preparation was mixed in an equal volume with either complete Freund's adjuvant (Gibco-BRL Burlington, ON) for the first injection or with incomplete Freund's adjuvant for subsequent injections, usually at 4 week intervals. The antigen-adjuvant mixtures were sonicated to prepare emulsions, immediately prior to injection of the mice. Blood was collected from the saphenous vein using a heparin coated capillary tube. ELISA screening of mouse polyclonal serum to ascertain the antibody titer was carried out before proceeding with the fusions (see below) (Tolson, 1989).

2.10 Enzyme-linked immunosorbent assay (ELISA)

Indirect enzyme-linked immunosorbent assay (ELISA) was used for screening of the mouse polyclonal anti-serum to ascertain the antibody titer before proceeding with the fusions (Tolson, 1989). In addition, indirect ELISA was used for screening of hybridoma supernatants.

In brief, flat-bottom ELISA plates (Falcon 3915 PRO-BIND™ Assay Plates, Becton-Dickinson, Oxnard, CA) were coated with 100 μL/well of lysed KO1 trypanosomes in water (2 X 10^7 cells/mL) and dried overnight at 37 °C. The primary antibody was polyclonal mouse antiserum diluted with PBS (doubling dilutions starting at 1:10). Control serum, similarly diluted, was from normal (unimmunized) BALB/c mice. The secondary antibody used was a 1:2000 dilution of alkaline-phosphatase-

conjugated goat anti-mouse IgG/IgM (Caltag, South San Fransisco, CA, USA). The substrate used was *p*-nitrophenol phosphate (Sigma Chemical Company, Mississauga, ON, Canada) and 100 µL were added to each well. The results from the plates were determined by reading the absorbance at 405 nm using an automated EIA plate reader (Model EL 310, Bio-Tek Instruments, Burlington, VT, USA).

2.11 Derivation of monoclonal antibodies

Derivation of hybridomas was performed as previously described using ClonaCell-HY™ (StemCell, Vancouver, BC.) for single step selection and cloning (Pearson, 1980; Richardson, 1986). After 10-14 days of growth in ClonaCell-HY™ individual hybridoma clones were picked into wells of 96-well microtitre tissue culture plates (Costar, Cambridge MA, USA) and grown for 2-4 days before 25 µL of hybridoma supernatant were carefully removed from the wells and screened by indirect ELISA as previously described above. The ELISA plates were coated with either live or glutaraldehyde-fixed wild type or EP/GPEET KO1 427.01 *T. b. brucei* PCF. ELISA plates coated with glutaraldehyde-fixed parasites were prepared as follows: trypanosomes harvested from log-phase cultures were washed with and resuspended to 4 x 10^7/ml in ice cold PBS containing 1% glucose. Fifty µL of this suspension were added to each well of flexible, round bottom polyvinyl chloride RIA microplates (Falcon 3911 Microtest III) and centrifuged at 50 x g for five min at 4 °C. One hundred µL of 25% fresh electron microscopy grade glutaraldehyde (EM Sciences, CEDARLANE) was gently added to each well and the plates were incubated at room temperature for 15 min. The plates were then centrifuged at 50 x g for 5 min at 4 °C. Supernatants were removed

by inverting the plates and gently flicking. The plates were then washed two times with ~100 μL PBS/well and removed by inverting and gently flicking. To prepare plates coated with live KO1 trypanosomes, cells were washed in ice cold PBS, resuspended to 5 X 10^6 cells/mL in distilled sterile water, and 100 μL of suspension were dispensed into 96 well flat-bottom ELISA plates (Falcon 3915 PRO-BIND™ Assay Plates, Becton-Dickinson, Oxnard, CA). The plates were then left overnight at 37 °C to dry. For these ELISAs, controls included: polyclonal antiserum from the immunized mouse diluted 1:300 with PBS-TWEEN/ 1% skim milk powder, serum from a non-immune mouse diluted 1:300 with PBS-TWEEN/ 1% skim milk powder, undiluted supernatant containing anti-human transferrin monoclonal antibody, undiluted supernatant containing anti-EP procyclin monoclonal antibody 247 (Richardson, 1988) undiluted supernatant from the parental myeloma X63 Ag8.653 and the diluent PBS-TWEEN.

2.12 Cryopreservation of hybridomas

After screening, selected hybridomas were frozen in liquid nitrogen. Freezing medium (90% FBS, 10% DMSO) was filter sterilized using a 0.2 μm syringe filter. Hybridomas from healthy, rapidly dividing cultures (50 mL) were centrifuged at 1000 X G for 6 min at room temperature. The supernatants were removed and the cells gently resuspended in 6 mL of freezing medium. The cells were then dispensed (1 mL each) into labeled cryovials and placed into a freezing head of a liquid nitrogen tank to be left over night to slowly cool before sinking the following day. Cell cultures were maintained until one vial had been thawed to ensure viability of the preserved cells.

2.13 Isotyping of monoclonal antibodies

Isotyping of selected monoclonal antibodies was performed using an antigen-capture ELISA kit following instructions supplied by the manufacturer (American Qualex, La Mirada, CA). Undiluted hybridoma tissue culture supernatants were used for the assay.

2.14 PEG purification of ascites mAb 1G10

Monoclonal antibody 1G10 was purified from murine ascites fluid using a procedure employing polyethylene glycol (PEG) (Masri, 1983). After centrifugation to remove any cellular debris, 10 mL of ascites fluid were added to 10 mL 80 mM potassium phosphate buffer, pH 7.0 and placed in a 50 mL conical tube with a magnetic stir bean. Diluted PEG-8000 (10 mL of 1:2 dilution in 80 mM potassium phosphate buffer) was added drop wise to the ascites solution using a buret until the final antibody solution contained 15% PEG. The sample was centrifuged at 3960 X G for 30 min at room temperature. The supernatant was removed and put aside, while the pellet was resuspended in 20 mL potassium phosphate buffer (30 mM, pH 7.0). The PEG precipitation was repeated with 7 mL 50% PEG added drop wise using a buret until the final antibody concentration contained 13% PEG. Both PEG precipitation steps were performed with stirring. The sample was centrifuged as described above and the supernatant removed. The pellets were resuspended in 1 X PBS, pH 7.4 to dissolve the pellet for dialysis. The sample was dialyzed against PBS to remove the PEG. The optical density (OD) (280 nm) of the purified antibody sample was used to determine the approximate protein concentration. For IgM immunoglobulins 1 mg/mL of protein is

equal to an OD at 280 nm of 1.2. The purified monoclonal antibody had an OD reading of 5.69 and thus an approximate protein concentration of 4.74 mg/mL.

2.15 Immunofluorescence microscopy and flow cytometry

Indirect immunofluorescence was performed on suspensions of live parasites as previously described (Pearson, 1981). Hybridoma tissue culture supernatants were used as the primary antibody, diluted in a 1:1 (v:v) ratio with 25 µL of 5 X 10^7 trypanosomes suspended in D-MEM medium. All steps were performed with medium and cells incubated on ice. After incubation for 30 min the cells were washed three times for 10 min with D-MEM medium. Then 50 µL of 1:400 dilution of Alexa Fluor 488-labeled goat anti-mouse IgG (H and L) secondary antibody in PBS (Molecular Probes, Eugene OR, USA) were added and the suspension was incubated for 30 min at room temperature. The trypanosomes were then washed as before. Cells were resuspended in ice cold PBS and 10 µL smeared onto poly-L-lysine coated slides. Immunofluorescence was observed using a Zeiss Standard microscope fitted with an epifluorescence attachment and an oil-immersion 100 X Neofluar objective (Carl Zeiss, Oberkochen, West Germany). Photographs were taken using a 3CDD DAGE-MTI model DC330 camera.

Immunofluorescence analysis of formaldehyde fixed trypanosomes was performed as follows: Twenty five mL of PBS were measured into a 50 mL conical tube and microwaved until the temperature reached 80-90 °C. One g of paraformaldehyde (SIGMA) was added and the tube was shaken violently until all of the paraformaldehyde was dissolved. The 4% paraformaldehyde was used immediately or aliquoted and frozen at -20 °C. Cells were washed and resuspended to 1 X 10^7 in 5 mL paraformaldehyde for

15 min at room temperature. The parasites (1 X 10^7) were then rinsed two times with 1 mL PBS and then blocked for 20 min. with PBS containing 0.1% Triton X-100 and 5% goat serum. The blocking buffer was removed and primary antibody (undiluted tissue culture supernatant) added and incubated for 30 min at room temperature. Cells were washed three times 10 min (no shaking) with PBS and then 50 µL 1:400 diluted Alexa Fluor 488-labeled goat anti-mouse IgG (H and L) secondary antibody in PBS (Molecular Probes, Eugene, OR, USA) was added and incubated for 30 min at room temperature. Cells were washed as before and resuspended in 20 µL PBS. Ten µL of the suspension were spotted onto a slide, fitted with a cover slip and cells were visualized by indirect immunofluorescence as described above.

For flow cytometric analysis, cells were resuspended in 500 µL FacsFlow sheath fluid and analyzed using a FACSCalibur™ flow cytometer (Becton-Dickinson, San Jose, CA). For every sample 10,000 events were recorded.

2.16 1-D and 2-D immunoblot analysis

Electroblotting of proteins from 1-D polyacrylamide gels onto BioTrace™ PVDF membrane (Pall Corporation, Ann Arbor, MI) was performed as described previously (Beecroft, 1993). Proteins were transferred from 1-D Bio-Rad mini-gels to PVDF membrane for 30 min at 90 V, with an ice pack in the transfer buffer outer chamber. Proteins separated by 2-D SDS-PAGE were transferred from the second dimension slab gels onto PVDF at 300 mA for 1.5 hours using a BIORAD electrophoretic transfer system (Bio-Rad Laboratories, Hercules, CA, USA.). The blot box was placed in a large bin filled with ice to surround the system and keep it cool for the duration of the transfer.

Fermentas 10-160 kDa pre-stained molecular mass markers were run on the gels to help verify protein transfer and to serve as molecular mass standards. Post-electrophoretic transfer, PVDF membranes were placed into blocking buffer (PBS/0.1% TWEEN-20/5% skim milk powder) overnight at 4 °C. All of the following steps were carried out at room temperature with agitation (rapid agitation for wash steps and slow, gentle agitation for incubation steps). Membranes were then washed and the primary antibody (undiluted hybridoma supernatant) was added and incubated at room temperature for one hour. After a third washing step the secondary antibody, diluted 1:50,000 anti-mouse IgG/IgM conjugated to horseradish peroxidase (Caltag Laboratories, South San Francisco, CA), was added and incubated for one hour. After washing the immunoblots were coated in SuperSignal Dura enhanced chemiluminescence substrate (Pierce Chemical Company, Rockford, IL) to detect the bound HRPO conjugated antibody. Membranes were then developed by exposure to Kodak Biomax MR film. All membranes were stained post-transfer with 0.1% Nigrosin/PBS to detect protein bands corresponding to the proteins separated by gel electrophoresis. The exposed film was then superimposed over the stained PVDF membrane to observe the location of immunoreactive protein bands in relation to the whole protein profile.

2.17 In-gel tryptic digestion

For analysis by matrix assisted lazer desorption-time of flight (MALDI-TOF) mass spectrometry, 2-D protein spots stained with colloidal Coomassie Brilliant Blue G-250 were cored using 200 µL yellow Eppendorf pipette tips cut 0.5 cm from the tip. The cored spots were transferred to 1.5 mL Eppendorf microcentrifuge tubes. These spots

were then de-stained (50 % v/v methanol/ 5 % v/v acetic acid), reduced with 10 mM DTT and alkylated with 100 mM iodoacetamide as described previously (Hanna, 2000). Following reduction and alkylation, protein spots were digested overnight at 37 °C with 20 ng/μL modified porcine sequence grade trypsin according to the manufacturer's directions (Promega, Madison, WI). Peptides were extracted from the gel pieces using one wash with 30 μL of 50 mM ammonium bicarbonate and 2 X 30 μL elutions 10% formic acid. The resulting pooled eluates were reduced to a final volume of 20 μL in a vacuum centrifuge prior to analysis by mass spectrometry.

2.18 Mass Spectrometry

Tryptic peptides from each sample were desalted using a ZipTip (C18 resin; P10, Millipore Corporation, Bedford, MA). For each sample, 1.0 μL of the desalted peptide mixture was mixed (1:1) with the matrix α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) and spotted onto a Voyager, 100 position, stainless steel MALDI plate (Applied Biosystems, Foster City, CA). An Applied Biosystems Voyager DE-STR mass spectrometer (Applied Biosystems, Foster City, CA) running in delayed extraction, reflectron mode was used to acquire MALDI-TOF data. Peptide mass profiles were analyzed using two different algorithms, MASCOT (Matrix Science) and MS-FIT (Protein Prospector), and searching two different data bases for alignments, NCBI and SWISS-PRO, in attempts to identify the protein.

2.19 Biotin labeling of cell surface proteins

Wild type, Nour 6 (*EP* knock out), KO1 (*EP/GPEET* knock out) and KO2 (*GPI10* knock out) PCF trypanosomes were surface labeled with Sulfo-N-hydroxysuccinimidyl (NHS)-biotin (PIERCE, Rockford, IL). In brief, 2.5 X 10^7 parasites harvested from log-phase were washed four times with ice cold PBS/0.1% glucose and then recounted using a hemocytometer. The cells were resuspended to 1 X 10^7 in 500 µL PBS and incubated with 2 mg of Sulfo-NHS-biotin for 30 min at room temperature. Cells were washed two times with PBS, centrifuged and resuspended in 25 µL 1X Laemmli buffer. Proteins were separated by SDS-PAGE and electroblotted to PVDF membrane as described above. The PVDF was incubated in blocking buffer overnight at 4 °C and then washed 5 X 10 min in wash buffer with shaking. Blots were incubated with 150 mL Streptavidin-HRPO (1:150,000 dilution in PBS/0.5% skim milk powder) for 30 min at room temperature. Blots were washed 3 X 10 min in washing buffer and developed with SuperSignal Dura enhanced chemiluminescence substrate and exposed to Kodak Biomax MR film. The film was exposed for 1 and 2 min intervals. The PVDF was then stained with 0.1% Nigrosin/PBS to detect the proteins present after transfer.

2.20 Avidin affinity chromatography

Wild type, Nour 6 (*EP* knock out), KO1 (*EP/GPEET* knock out) and KO2 (*GPI10* knock out) PCF trypanosomes were washed four times in PBS/0.1% glucose and resuspended to a concentration of 2.5 X 10^7 cells in PBS/5 mg/mL biotin for 30 minutes at room temperature. The cells were washed two times in PBS and then resuspended in 1 mL 1% NP40 / 1X protease inhibitor cocktail/ 1X PMSF for 30 minutes at 4 °C with

mixing by rotation. The protease inhibitor cocktail (PIC) contained 500 μM AEBSF, HCl, 150 nM A protein, 1 μM E-64, 1 μM leupeptin, hemisulfate and 1 μM PMSF. Avidin beads were washed 5 times with PBS and resuspended in PBS (1:1 v/v) before use. Cells were pelleted at 140 X g for 5 minutes and then resuspended with 150 μL avidin beads in an equal volume of PBS plus 1X PIC. After incubation for 30 minutes at room temperature with rotation, the beads were washed with a 1% NP40 solution for 2 X 1 min, 4 X 5 min and 2 X 2 min. The beads were resuspended in 40 μL 1X Laemmli sample buffer and boiled for 8 minutes. The supernatant was loaded into wells of a 1-D SDS-PAGE gel, both undiluted and 50% diluted in 1X Laemmli sample buffer. Proteins separated on the gel were transferred to PVDF membranes by electroblotting and biotin labeled proteins were detected as described previously using Strepavidin-HRPO.

Wild type and KO1 trypanosomes were biotin labeled for 2-D gel and immunoblot analysis as previously described for 1D analysis except for the following amendments to the protocol. Wild type (2×10^8) and KO1 (4×10^8) cells were incubated with 150 μg biotin in 2 mL PBS plus PIC. After the incubation and washing steps, pelleted cells were resuspended and mixed with 250 μL avidin beads in an equal volume of PBS containing 1X PIC. The beads were incubated in 40 μL urea mix and loaded onto the first dimension isoelectric focusing gels. After separation in the second dimension, the gels were fixed and stained with colloidal Coomassie Brilliant Blue G-250 to detect proteins eluted from the avidin beads. Protein spots were cored and analyzed using MALDI-TOF mass spectrometry to identify the affinity-isolated biotinylated proteins.

3. Results

3.1 1D and 2D protein profiles of wild type and mutant *T. b. brucei* PCF

The differences in protein expression between wild type and three different surface mutants of *T. b. brucei* PCF trypanosomes have not been examined before. The protein profiles of wild type *T. b. brucei* 427.01 PCF were compared to those of three PCF mutants: Nour 6 (*EP* knock out), KO1 (*EP/GPEET* knock out) and KO2 (*GPI10* knock out). Proteins in lysates from all four PCF trypanosomes were separated by 1D SDS-PAGE and stained with colloidal Coomassie Brilliant Blue G-250 or silver. The protein profiles of all four parasites appeared to be indistinguishable when stained with colloidal Coomassie Brilliant Blue G-250 **(Figure 6)**, and no clear differences could be detected between the wild type and any of the mutants. Silver staining increases sensitivity of protein detection by 10-100 fold, however the mutants were the same as wild type results, except many of the bands appeared much darker on the silver stained gel **(Figure 7)**. Proteins from all four PCF trypanosomes were also separated by 2D SDS-PAGE and detected by staining with colloidal Coomassie Brilliant Blue G-250 **(Figure 8)**. A light box was used to visually compare the 2D gel protein profiles all four PCFs. All four were very similar to each other.

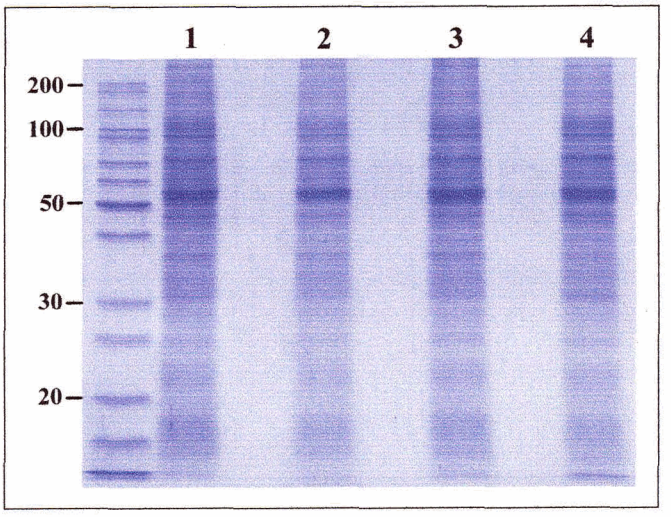

Figure 8. One-dimensional gel electrophoretic separation of proteins of wild type and mutant *T. b. brucei* PCF. Proteins from 2 X 10^6 cells were separated by 1D SDS-PAGE and detected by staining with colloidal Coomassie Brilliant Blue G-250. Lane 1. Wild type 427.01 PCF. Lane 2. Nour 6 (*EP* knock out) 427.01 PCF. Lane 3. KO1 (*EP/GPEET* knock out) 427.01 PCF. Lane 4. KO2 (*GPI10* knock out) 427.01 PCF. Molecular mass standards (kiloDaltons) are shown on the left side of the gel.

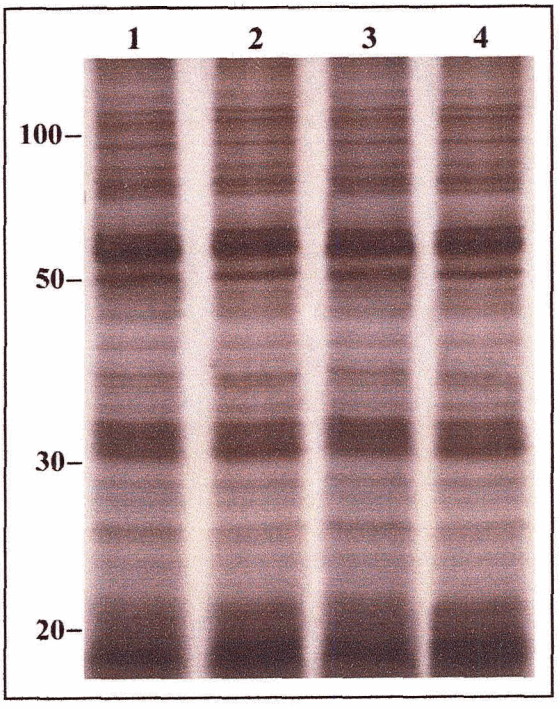

Figure 9. One dimensional gel electrophoretic separation of proteins of wild type and mutant *T. b. brucei* PCF. Proteins from 2 X 10^6 cells were separated by 1D SDS-PAGE and detected by silver staining. Lane 1. Wild type 427.01 PCF. Lane 2. Nour 6 (*EP* knock out) 427.01 PCF. Lane 3. KO1 (*EP/GPEET* knock out) 427.01 PCF. Lane 4. KO2 (*GPI10* knock out) 427.01 PCF. Molecular mass standards (kiloDaltons) are shown on the left side of the gel.

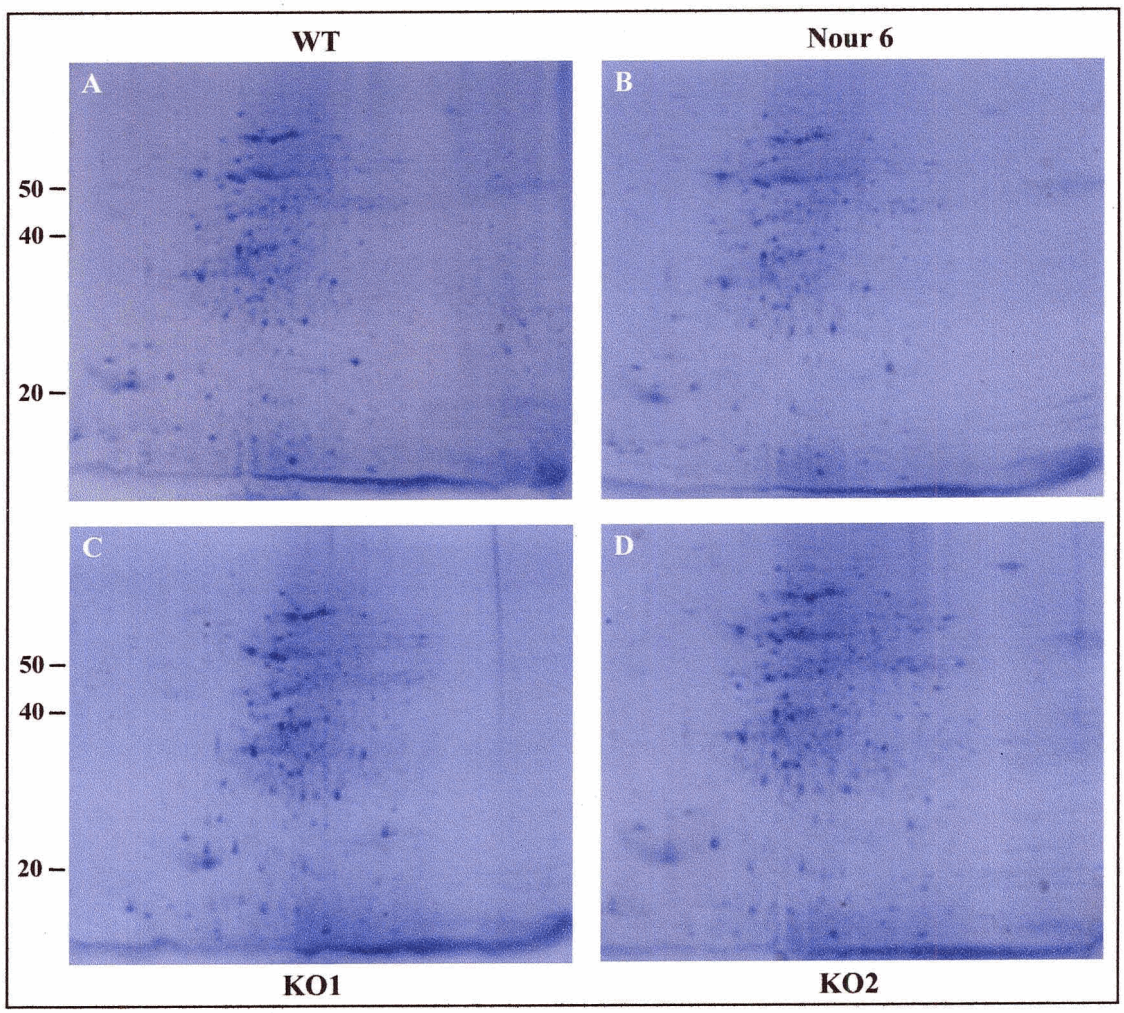

Figure 10. Two-dimensional polyacrylamide gel electrophoresis of proteins from wild type and mutant *T. b. brucei* PCF. Proteins from 2 X 10^7 cells of each clone were solubilized, separated and stained with colloidal Coomassie Blue G-250. Wide range ampholines pH 3-10 were used in the first dimension IEF and a 10-15% gradient SDS-polyacrylamide gel for the second dimension. Panel A. Wild type 427.01. Panel B. Nour 6 (*EP* knock out) 427.01 PCF. Panel C. KO1 (*EP/GPEET* knock out) 427.01 PCF. Panel D. KO2 (*GPI10* knock out) 427.01 PCF. The gels are shown with the basic end to the right of each panel and molecular mass markers (kiloDaltons) are shown on the left side of the diagram.

3.2 Membrane preparation from *EP/GPEET* KO1 trypanosomes

A crude membrane extract was prepared from KO1 PCF and the proteins were analyzed via SDS-PAGE analysis and detected by staining with colloidal Coomassie Blue **(Figure 9)**. The protein profiles showed a lot of similarity except the proteins in the 1.25 g/mL band were more concentrated than those in the 1.17 g/mL band. A BCA assay was used to generate a standard curve from which an approximate protein concentration for each fraction was determined by extrapolation **(Figure 10)**. The BCA assay confirmed that the 1.25 g/mL fraction was more concentrated and contained about 9.2 μg/μL of protein while the 1.17 g/mL fraction contained about 5.6 μg/μL of protein.

3.3 Derivation of monoclonal antibodies

Four mice were immunized with either live PCF parasites or membrane preparations from KO1 PCF. Just prior fusions to generate hybridomas the antibody titre in the serum of each mouse was tested against lysed KO1 trypanosomes via indirect ELISA. The antibody titres in the sera of mouse 1 and 2 from Group A were both higher than the non-immunized mouse serum used as the negative control **(Figure 11)**. These mice were sacrificed shortly after this assay and the spleenocytes removed from the spleen for hybridoma production.

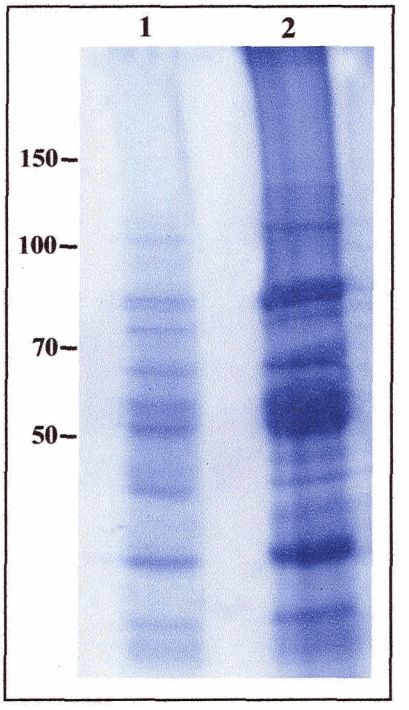

Figure 11. One-dimensional gel electrophoretic separation of membrane proteins extracted from *T. b. brucei* KO1 knock out PCF. Proteins were separated from the 1.17 g/mL and 1.25 g/mL bands in the sucrose density gradient and were detected by staining with colloidal Coomassie Brilliant Blue G-250. Lane 1. Proteins from 1.17 g/mL band. Lane 2. Proteins from 1.25 g/mL band. Molecular mass standards (kiloDaltons) are shown on the left side of the gel.

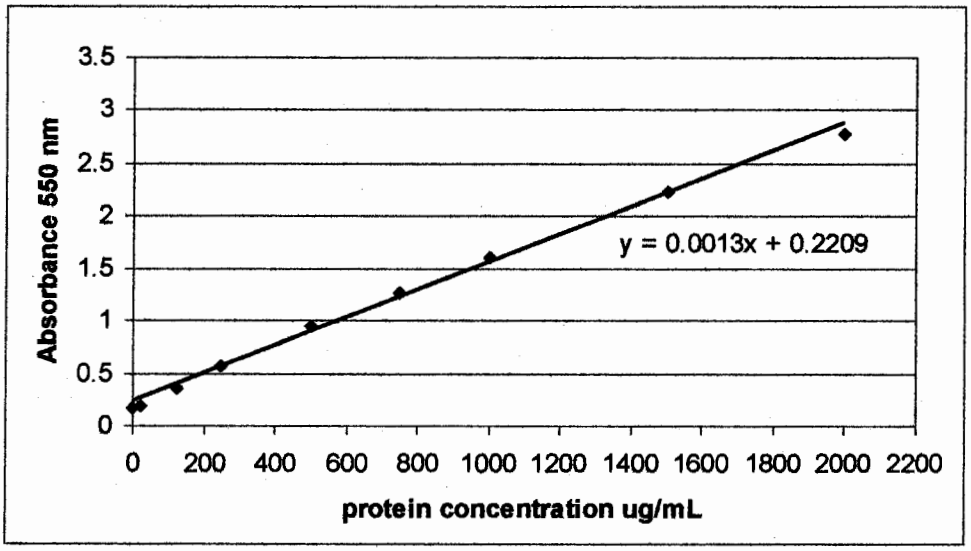

Figure 12. Protein quantification of membrane preparations of *T. b. brucei* PCF. A BCA assay was used to determine the protein concentration of membrane preparation samples. The 1.17 g/mL and 1.25 g/mL bands extracted from the sucrose density gradient were dialyzed with PBS, concentrated and desalted. The approximate protein concentrations of the final samples were extrapolated from the standard curve generated using the BCA assay. The protein concentration in the 1.17 g/mL sample was approximately 5.6 µg/µL and in the 1.25 g/mL sample was approximately 9.2 µg/µL.

3.4 Selection of monoclonal antibodies

Approximately 250 hybridoma clones were picked from each cell fusion experiment (immunized with either live KO1 PCF or their membrane extracts). Indirect ELISA assays were used to detect positive clones, meaning they produced mAbs that recognized wild type and/or KO1 antigens. Using the ELISA assay a positive result was an absorbance reading at 405nm that was at least 2 or 3 times higher than the absorbance at 405 nm of the negative control (non-immune, mouse serum). The positive clones from this first round of screening were then tested on human transferrin coated ELISA plates to eliminate mAbs that bind non-specifically. Based on this initial screening, the mAbs 1G10 and 2E1 were selected for further analysis. Both of these mAbs were isotyped as IgM immunoglobulins.

3.5 Immunofluorescence microscopy and flow cytometric analysis of mAb 1G10 and mAb 2E1

Wild type, KO1 (*EP/GPEET* knock out) and KO2 (*GPI10* knock out) PCF trypanosomes were labeled with mAbs 1G10 and 2E1 for flow cytometry and immunofluorescence microscopy. Cells were either live or fixed with paraformaldehyde and were labeled with the antibodies to determine the presence and location of their relevant antigens. The flow cytometry and immunofluorescence microscopy experiments were performed with positive and negative controls and each experiment was repeated 8-10 times to ensure that the results repeated. Live PCF trypanosomes were labeled with either mAb 1G10 or 2E1 for flow cytometry and 10,000 events were recorded to generate histograms of parasite fluorescence **(Figure 12)**. The positive control (wild type PCF

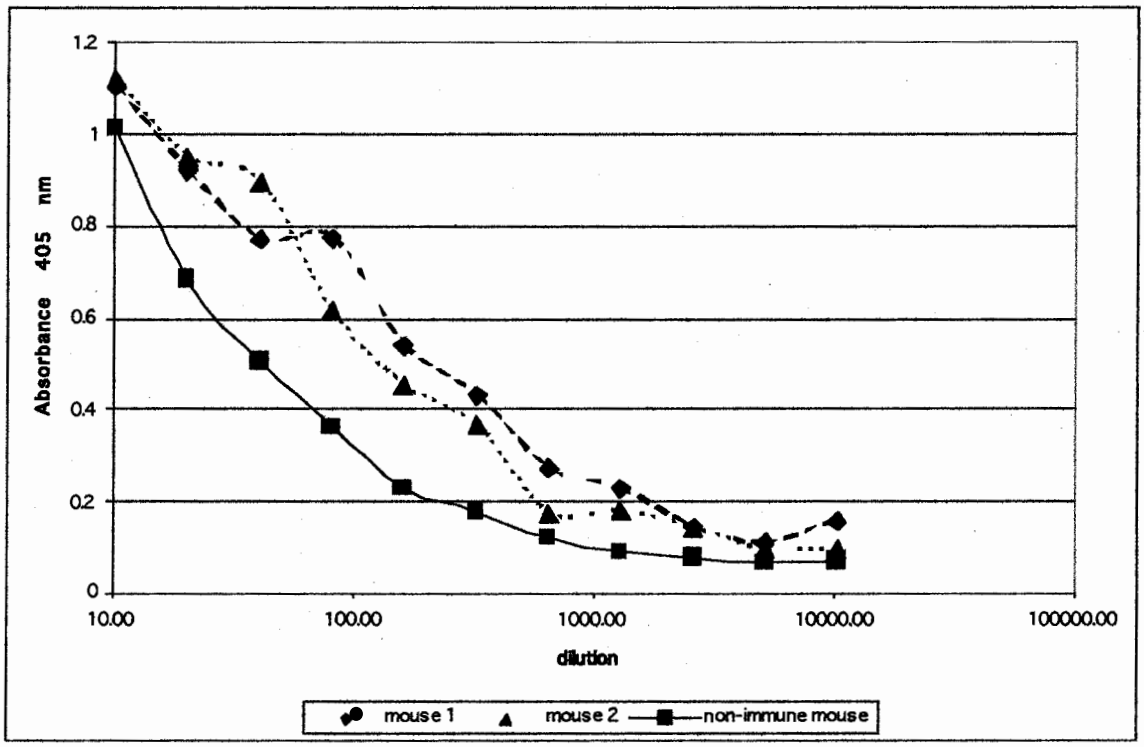

Figure 13. Indirect ELISA titration of test bleed serum from Group A mice immunized with live KO1 (*EP/GPEET* knock out) parasites. Mouse serum was titrated against lysed KO1 cells dried onto ELISA microplate wells. Lysed KO1 trypanosomes (2 X 10^7 cells/mL) were distributed into each well of a 96 well ELISA plate and dried overnight. Antibodies in the serum were detected by reading the absorbance at 405 nm. The legend is shown at the bottom of the panel. The control serum from a non-immune mouse is shown as a solid line with black squares. The test sera from the two immunized mice are shown as the broken and dotted lines.

labeled with anti-EP mAb 247) showed 1000 fold greater fluorescence than the negative control (KO1 PCF labeled with anti-EP mAb 247) **(B)**. Anti-KO1 mAb 1G10 showed no labeling of either KO1 or wild type PCFs (**C, D** respectively). Anti-KO1 mAb 2E1 showed no labeling of KO1 PCF **(E)** but labeled wild type PCF as seen by the 100-1000 fold greater fluorescence than the negative control **(F)**. Parasites were also fixed with paraformaldehyde and labeled with the same mAbs to determine whether or not fixation would allow antibody-antigen interaction. When cells were paraformaldehyde fixed mAb 1G10 and mAb 2E1 labeled antigens on wild type and KO1 PCF trypanosomes **(Figure 13)**. The positive control **(A;** wild type cells labeled with anti-EP mAb 247) showed 10-100 fold greater fluorescence than the negative control (**A-E;** KO1 knock out PCF labeled with anti-EP mAb 247). Labeling of fixed wild type and KO1 knock out PCFs with mAbs 2E1 and 1G10 showed similar amounts of fluorescence when compared to the positive control.

Live and fixed wild type, KO1 and KO2 PCF trypanosomes were labeled with three different mAbs and the immunofluorescence images were captured by a CCD camera attached to an epifluorescent microscope. The different PCF trypanosomes were labeled with mAb 247 (anti-EP), mAb 2E1 (anti-KO1) and mAb 1G10 (anti-KO1). Live PCFs did not show any labeling of antigen with mAbs 1G10 and 2E1 and therefore those images are not shown. However, fluorescence of fixed parasites produced very different results, consistent with the flow cytometric results **(Figure 14)**. Wild type PCFs were uniformly labeled over the entire cell surface with anti-EP mAb 247 (positive control; **A**), in contrast to the complete lack of fluorescence seen with KO1 knock out PCFs labeled

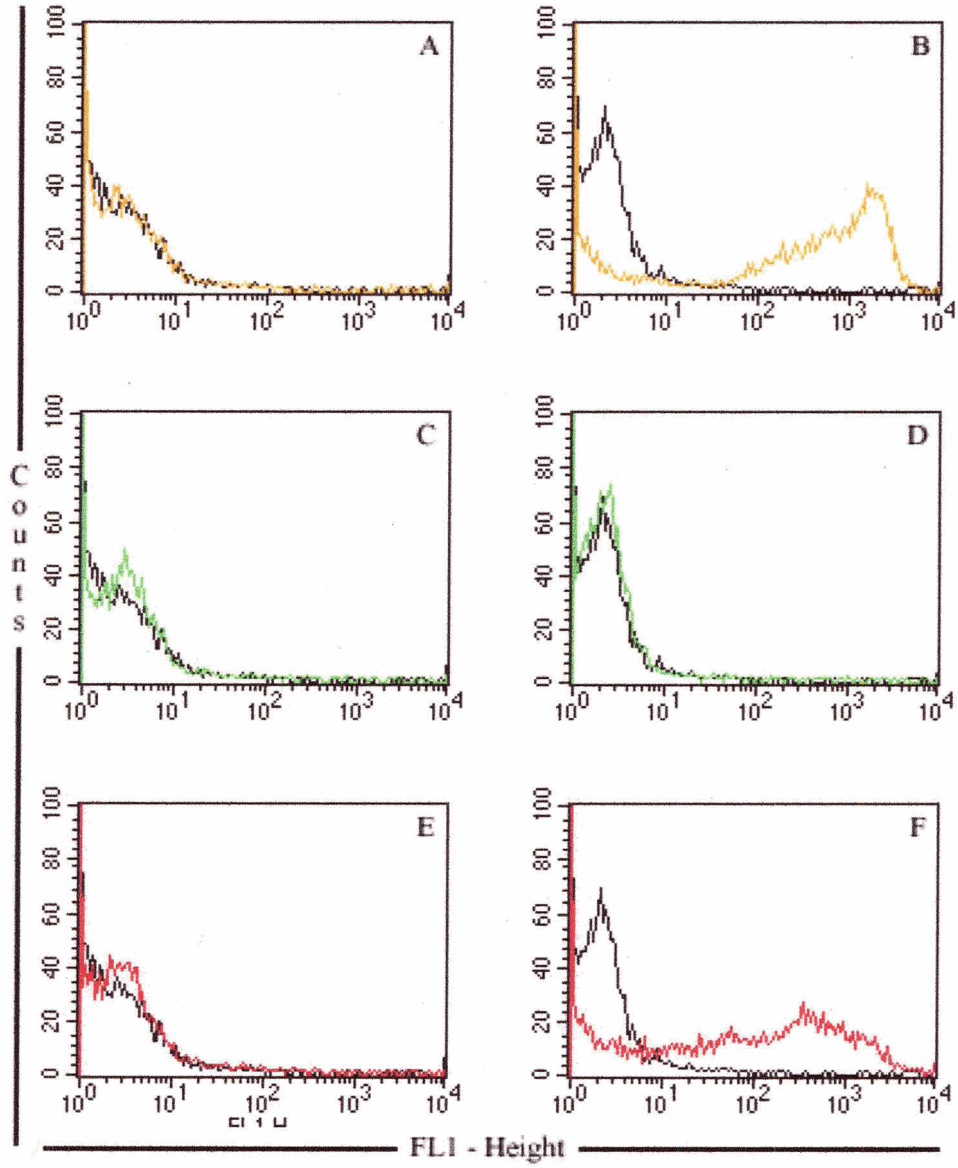

Figure 14. Flow cytometric analysis of live wild type and KO1 knock out mutant *T. b. brucei* PCF labeled with anti-KO1 mAbs 1G10 and 2E1. Trypanosomes (1 X 10^8 cells/mL) were fixed with 4% paraformaldehyde immediately prior to labeling with mAbs and flow cytometric analysis. The secondary antibody was Alexa Fluor 488-labeled goat anti-mouse IgG (H and L) diluted 1:400 in PBS. Panel A. Orange profile; KO1 PCF labeled with anti-EP mAb 247 (negative control). Panel B. Orange profile; wild type PCF labeled with anti-EP mAb 247 to label surface EP procyclins (positive control). Panel C. Green profile; KO1 PCF labeled with mAb 1G10. Panel D. Green profile; wild type PCF labeled with mAb 1G10. Panel E. Red profile; KO1 PCF labeled with mAb 2E1. Panel F. Red profile; wild type PCF labeled with mAb 2E1. Panels A, C, E. Black profile; KO1 PCF labeled with anti-human transferrin mAb as a negative control. Panels B, D, F. Black profile; wild type PCF labeled with anti-human transferrin mAb as a negative control. 10,000 events were recorded for each sample.

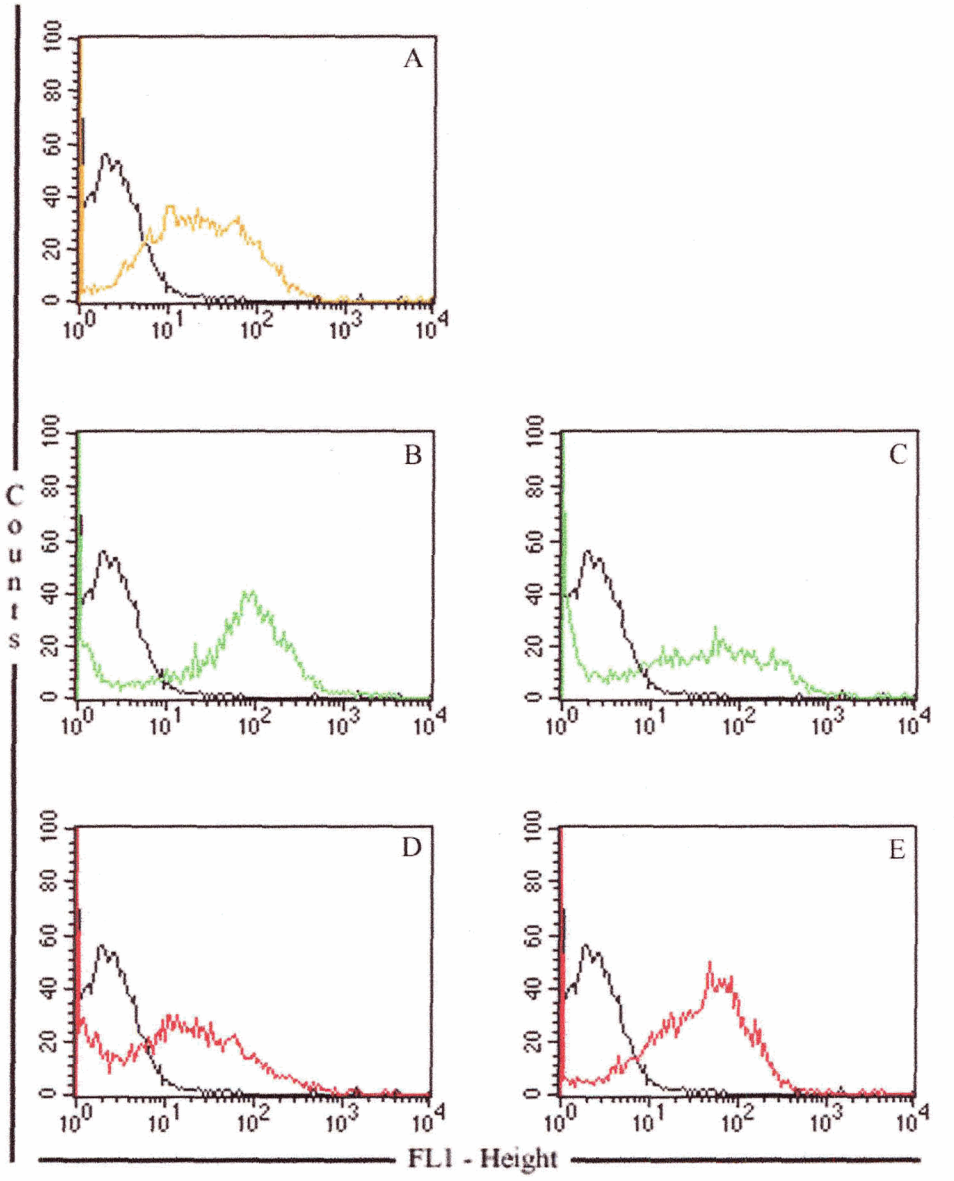

Figure 15. Flow cytometric analysis of live wild type and KO1 knock out mutant *T. b. brucei* PCF labeled with anti-KO1 mAbs 1G10 and 2E1. Trypanosomes (10 µL of 1 X 10^7 cells/mL) were used in each sample. The secondary antibody was Alexa Fluor 488-labeled goat anti-mouse IgG (H and L) diluted 1:400 in PBS. Panel A. Orange profile; wild type PCF labeled with anti-EP mAb 247 to label surface EP procyclins (positive control). Panel B. Green profile; KO1 PCF labeled with mAb 1G10. Panel C. Green profile; wild type PCF labeled with mAb 1G10. Panel D. Red profile; KO1 PCF labeled with mAb 2E1. Panel E. Red profile; wild type PCF labeled with mAb 2E1. Panel A-E. Black profile; KO1 PCF labeled with anti-EP mAb 247 as negative control. 10,000 events were recorded for each sample.

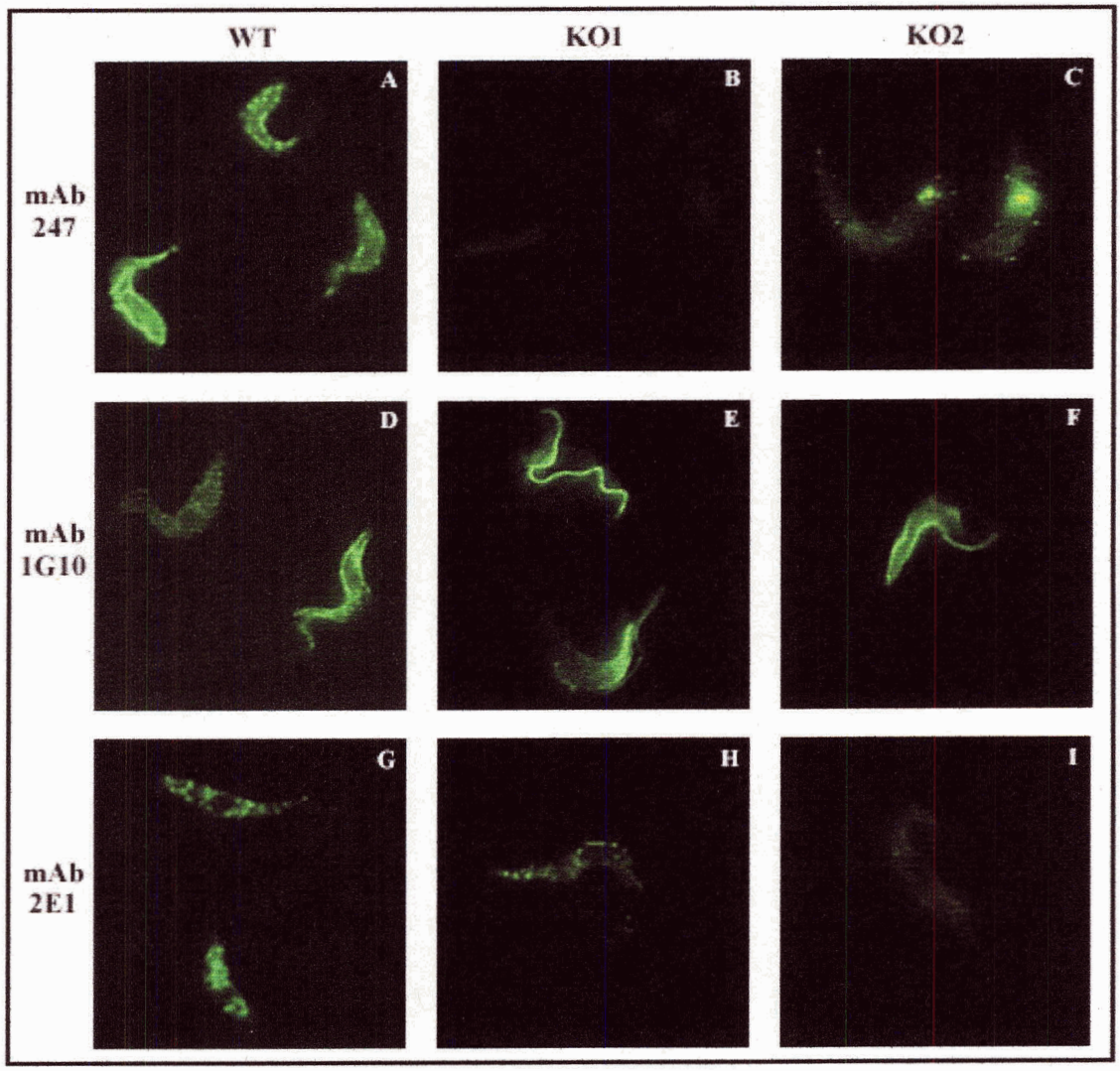

Figure 16. Immunofluorescence images of fixed wild type, KO1 (*EP/GPEET* knockout) and KO2 (*GPI10* knock out) *T. b. brucei* PCF labeled with anti-KO1 (*EP/GPEET* knock out) *T. b. brucei* PCF monoclonal antibodies (mAb) 1G10 and 2E1. All parasites were fixed with 4% paraformaldehyde immediately prior to labeling. Anti-EP mAb 247 was used as a positive control on wild type parasites (A) and as a negative control on *EP/GPEET* KO1 parasites (B).

with mAb 247 (negative control; **B**). KO2 PCFs, which do not express membrane bound procyclins, were positive for antigen labeling with mAb 247 **(C)**, mainly concentrated in one area of the parasite indicating that fixing with paraformaldehyde enabled mAb 247 to enter the cell and label procyclins inside. This suggested that KO2 knock out trypanosomes still expressed procyclins but were unable to anchor them in the membrane due to the *GPI10* knock out. Instead the proteins were retained within the cell. The images of wild type, KO1 and KO2 PCFs labeled with mAb 1G10 were very similar **(Figure 14; D, E, F)**. MAb 1G10 labeled an antigen that appeared to be present on the entire parasite surface (including the flagellum) on all three parasites. The fluorescence was uniform over the entire cell however it appeared to be more intense along the sides of the parasites and the flagella. MAb 2E1 on the other hand only labeled antigens on wild type and KO1 PCFs and the fluorescence was more diffuse and created a punctate pattern on the cell **(G, H)**. KO2 PCFs were not labeled by mAb 2E1 **(I)** suggesting that these parasites did not express the antigen recognized by 2E1 on wild type and KO1 PCFs.

3.6 1D and 2D immunoblot analysis using mAbs 1G10 and 2E1

It was important to determine whether or not mAb 1G10 and mAb 2E1 exhibited immunoblot reactivity. Proteins from both wild type and *EP/GPEET* KO1 trypanosomes were separated by 1D SDS-PAGE and transferred to PVDF membrane. The autoluminogram of this immunoblot showed that mAb 2E1 recognized a 50 kDa protein in KO1 PCF but did not recognize a protein in wild type PCF **(Figure 15)**. MAb 1G10 labeled a protein of about 55 kDa in both wild type and KO1 PCF **(Figure 15)**. Nigrosin staining of the PVDF membrane after immunoblot analysis enabled visualization of the

proteins that had transferred from the 1D gel (not shown). This ensured that the electrophoretic transfer had worked, and equal amounts of protein were present in each lane.

To isolate the antigens recognized by mAbs 1G10 and 2E1, proteins from both wild type and KO1 PCF trypanosomes were separated by 2D gel electrophoresis and transferred to PVDF membrane for immunoblot analysis. MAb 2E1 did not recognize any antigen(s) on 2D blots of either wild type or KO1 PCF protein lysates that had been separated by 2D SDS-PAGE (data not shown). However, mAb 1G10 strongly labeled a protein on immunoblots of both wild type and KO1 PCF lysates **(Figure 16)**. The autoluminogram of wild type proteins labeled with mAb 1G10 showed a horizontal smear (left to right) at about 55 kDa, a similar molecular weight to the protein recognized by mAb 1G10 on the 1D immunoblot **(Figure 15)**. Therefore a 2D gel of wild type proteins, run concurrently with the 2D gel used for immunoblot analysis, was stained with colloidal Coomassie Blue G-250 to detect the protein spots detected by mAb 1G10 **(Figure 17)**.

3.7 MALDI-TOF mass spectrometry analysis of antigens recognized by mAb 1G10

In order to isolate the antigen(s) recognized by mAb 1G10, the region labeled by mAb 1G10 on the 2D immunoblot **(Figure 16)** was aligned with a colloidal Coomassie Blue stained 2D gel of wild type PCF proteins **(Figure 17)**. Outlined by white circles and numbered 1-9 (from most acidic to least acidic), were the spots extracted from the 2D gel in order to identify the antigen(s) recognized by mAb 1G10 using MALDI-TOF mass spectrometry.

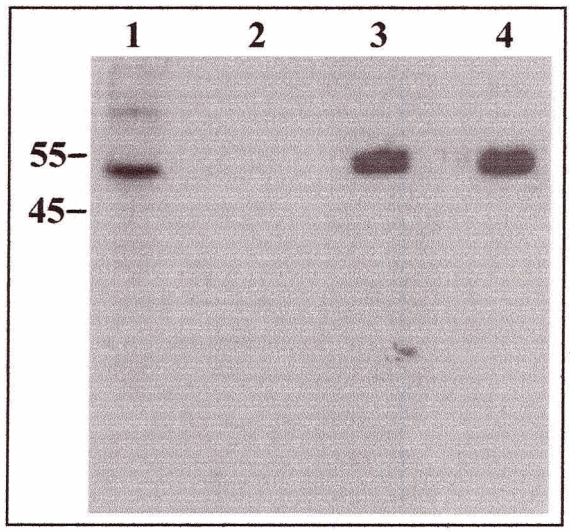

Figure 17. Immunoblot analysis of wild type and KO1 knock out mutant *T. b. brucei* PCFs using mAbs 2E1 and 1G10. Proteins of wild type and KO1 mutants were separated by 1D SDS-PAGE and transferred to PVDF membrane for immunoblot analysis. Lane 1. KO1 knock out 427.01 PCF. Lane 2. Wild type PCF. Lane 3. KO1 knock out 427.01 PCF. Lane 4. Wild type 427.01 PCF. Lane 1 and 2. mAb 2E1 raised against KO1 (*EP/GPEET* knock out) PCF. Lane 3 and 4. mAb 1G10 raised against KO1 (*EP/GPEET* knock out) PCF. Autoluminograms were developed after 10 sec exposure. Molecular mass standards (kiloDaltons) are shown on the left side of the blot.

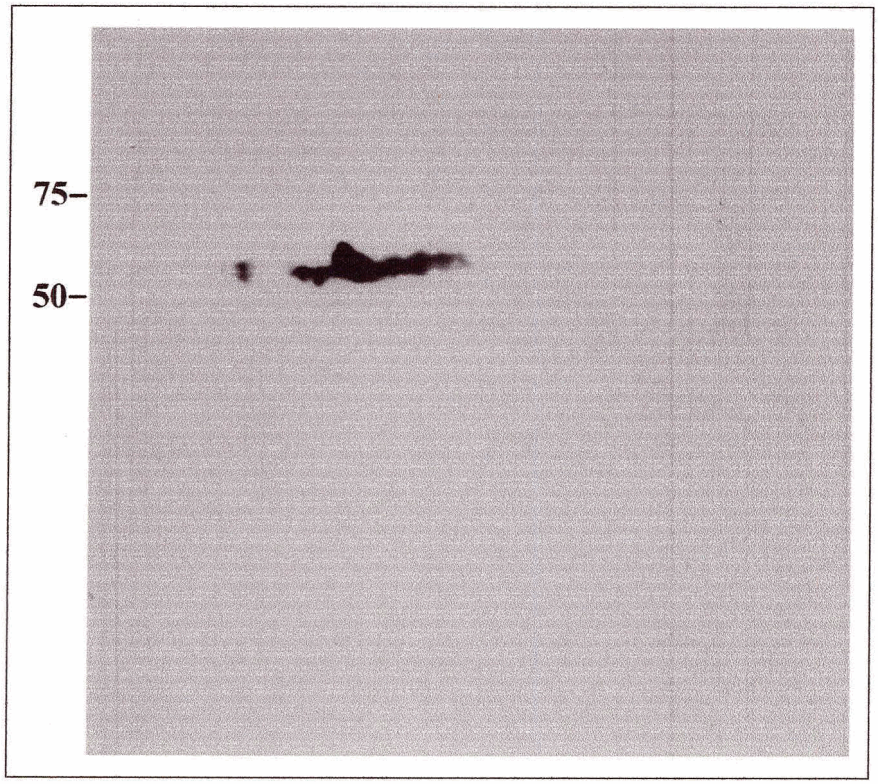

Figure 18. Detection of a 55 kDa protein in wild type *T. b. brucei* PCF after 2D gel electrophoresis and immunoblotting with mAb 1G10. Proteins from 2.5 X 10^7 cells were separated by 2D gel electrophoresis and then electroblotted onto PVDF membrane for immunoblot analysis. The primary antibody used was mAb 1G10 ascites (1:20,000). dilution with PBS/5% skim milk powder). The basic end of the gel is to the right of the autoluminogram. Molecular mass standards (kiloDaltons) are shown on the left side of the autoluminogram.

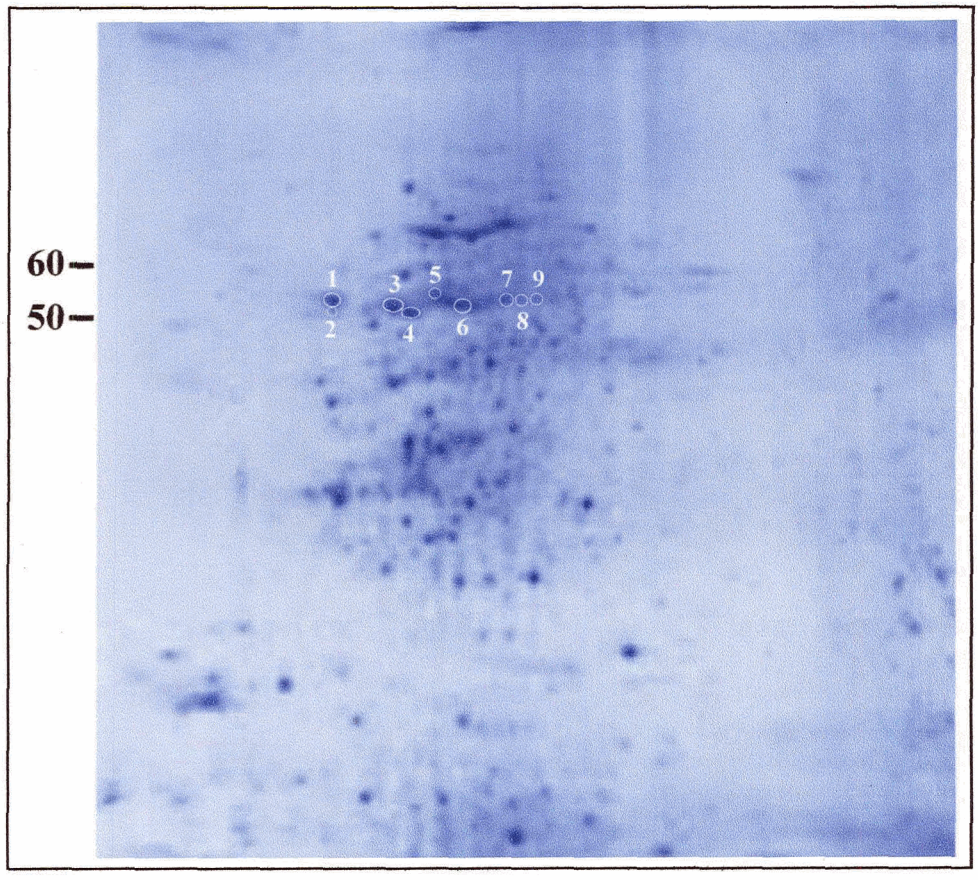

Figure 19. Two-dimensional gel profile showing protein spots extracted for tryptic digestion and MALDI-TOF analysis to identify the antigen recognized by mAb 1G10. Proteins from 2 X 10^7 wild type PCF trypanosomes were separated by 2D gel electrophoresis and proteins were detected by staining with colloidal Coomassie Brilliant Blue G-250. Wide range ampholines (pH 3–10) were used in the first dimension IEF, and a 10-15% gradient SDS-polyacrylamide gel for the second dimension. The spots analyzed by MALDI-TOF mass spectrometry are outlined with white circles on the gel. The gel is shown with the basic end to the right and molecular mass markers (kiloDaltons) are shown on the left.

These 9 spots produced peptide mass profiles that were analyzed using the Protein Prospector algorithm, MS-FIT, and by searching two data bases for mass matches: NCBI and SWISS-PROT. An example is shown of a peptide mass spectrum produced from a processed spot **(Figure 18)**. All of the spots produced peptides with mass values that matched peptide mass values in the database from tubulin of *T. b. rhodesiense* **(Table 1)**. The most acidic protein spots in the smear of protein recognized by mAb 1G10 on the immunoblot, #1 and #2, contained peptides with masses that matched those in the database from β-tubulin of *T. b. rhodesiense*, with a predicted molecular weight of 49.7 kDa and a predicted isoelectric point (pI) of 4.7. The least acidic spots, #5-9, , contained peptides with masses that matched those in the database from α-tubulin from *T. b. rhodesiense*, with a predicted molecular weight of 49.8 kDa and a predicted pI of 4.9. It was interesting that the two spots in the middle of the smear, #3 and #4, contained peptides that matched peptide masses from both α- and β-tubulin of *T. b. rhodesiense*.

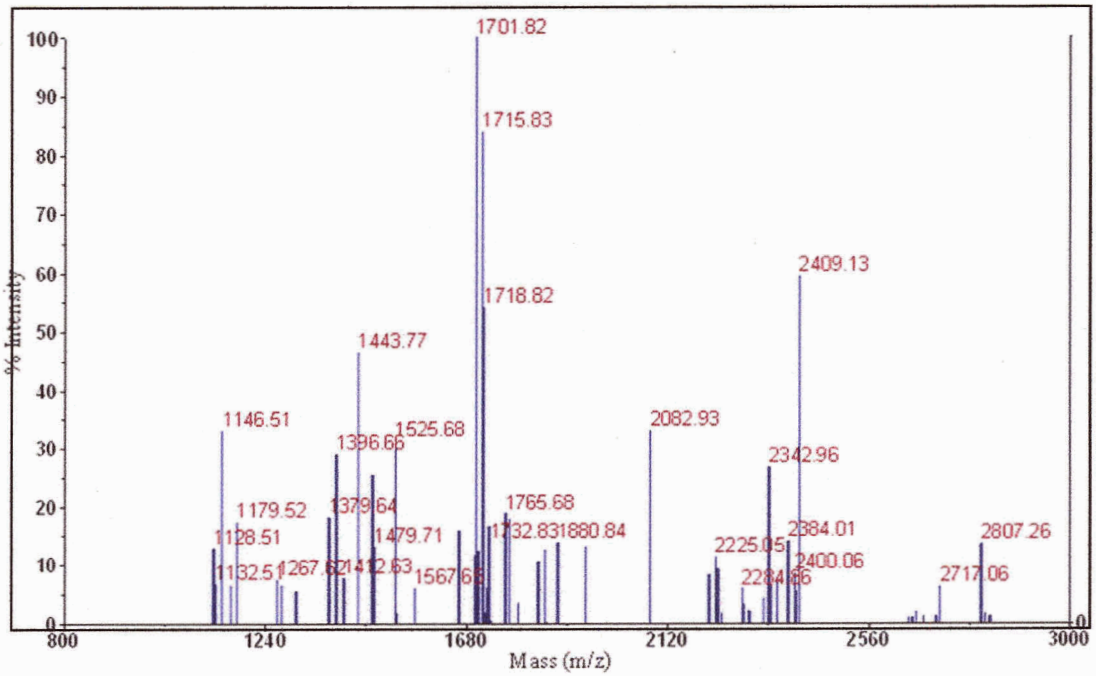

Figure 20. Peptide mass spectrum of the 55 kDa protein recognized by mAb 1G10. Spots from a colloidal Coomassie Blue G-250 stained 2D gel, (that aligned with the spots recognized by mAb 1G10 on the 2D immunoblot) were processed for MALDI-TOF mass spectrometry. Nine spots were extracted from the 2D gel and analyzed. Using the algorithm MS-FIT all of these spots produced peptide mass maps that aligned with either α- or β-tubulin from *T. b. rhodesiense*. Results from spot #7 are shown here.

Table 1. Summary of proteins identified by MALDI-TOF mass spectrometric analysis of protein spots recognized by mAb 1G10.

2D gel spot number	Protein ID[a] (Genbank accession No.)	MW[b] (kDa)	pI[c]
1	β-tubulin (71588)	49.7	4.7
2	β-tubulin (71588)	49.7	4.7
3	α-tubulin (71581)	49.8	4.9
4	α-tubulin (71581)	49.8	4.9
5	α-tubulin and β-tubulin (71581, 71588)	49.8, 49.8	4.9, 4.7
6	α-tubulin (71581)	49.8	4.9
7	α-tubulin (71581)	49.8	4.9
8	α-tubulin (71581)	49.8	4.9
9	α-tubulin (71581)	49.8	4.9

[a]Protein in the NCBInr 2005.01.06 database to which significant peptide mass matching was observed. Genbank accession numbers are provided.

[b]Predicted values determined by Protein Prospector search algorithm (MS-FIT)

[c]Predicted values determined by Protein Prospector search algorithm (MS-FIT)

3.8 Specificity of mAb 1G10

It was important to determine whether or not mAb 1G10 recognized tubulin in both life cycle stages of trypanosomes. Proteins from wild type, KO1 (*EP/GPEET* knock out) PCF, KO2 (*GPI10* knockout) PCF, and bloodstream form 427.01 trypanosomes were separated by 1D SDS-PAGE and transferred to PVDF for immunoblot analysis with mAb 1G10 **(Figure 19; A)**. MAb 1G10 detected tubulin of approximately 55 kDa in both procyclic culture forms and bloodstream form trypanosomes. This indicated that mAb 1G10 was not stage specific and recognized tubulin in parasites infective in both the mammalian host and insect vector. It was then imperative to investigate whether or not mAb 1G10 was an antibody that could be used to recognize tubulin in other kinetoplastids as well as mammalian cells. Proteins from different kinetoplastids, including different trypanosome species and Leishmania species, were separated by 1D SDS-PAGE along with mouse thymoma cells (EL4 cells) and human cervical epithelial carcinoma cells (HeLa cells). The proteins were transferred to PVDF for immunoblot analysis with mAb 1G10. This autoluminogram showed that mAb 1G10 labeled tubulin protein of about 55 kDa in each kinetoplastid protein analyzed but not in the mouse and human cells **(Figure 19; B)**. This suggested that mAb 1G10 only recognized tubulin protein expressed by kinetoplastids and not mammalian cells.

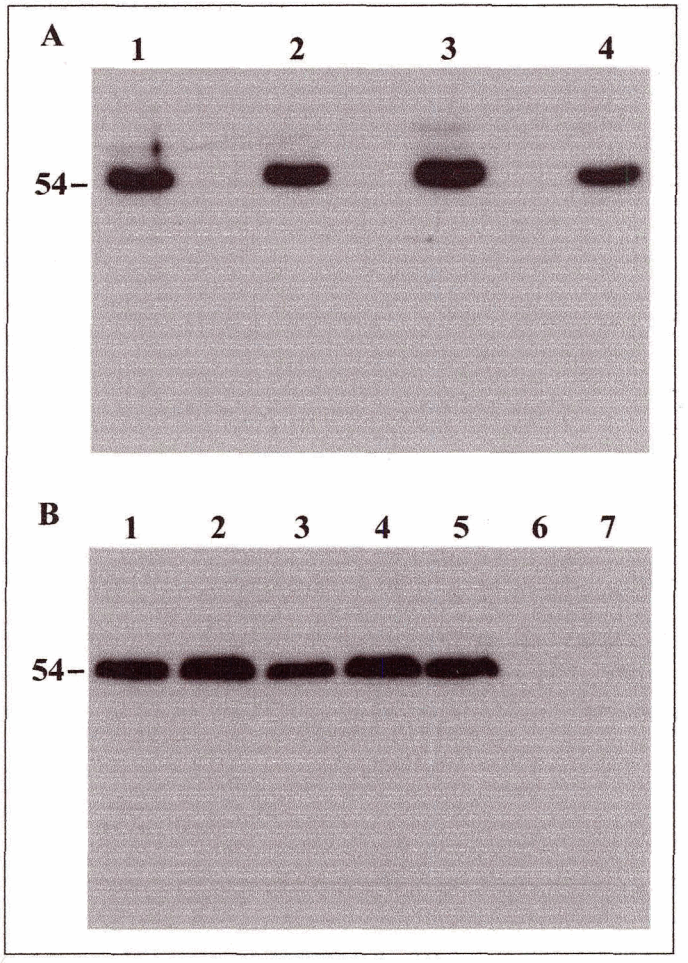

Figure 21. Detection of tubulin in wild type and mutant trypanosomes by immunoblotting with mAb 1G10. Trypanosome proteins were separated by 1D SDS-PAGE and then electroblotted onto PVDF membrane for immunoblot analysis. Panel A. *T. b. brucei* series. Lane 1. Wild type 427.01 PCF. Lane 2. KO1 (*EP/GPEET* knock out) 427.01 PCF. Lane 3. KO2 (*GPI10* knock out) 427.01 PCF. Lane 4. Bloodstream form 427.01 Clone 1. Panel B. Kinetoplastid series. Lane 1. Wild type 427.01 PCF. Lane 2. *Trypanosoma simiae* CP-11 PCF. Lane 3. *Trypanosoma congolense* IL-3000 PCF. Lane 4. *Leishmania donovani* promastigotes. Lane 5. *Leishmania major* promastigotes. Lane 6. Mouse EL4 cells. Lane 7. Human HeLa cells. Proteins from parasites and cells (5 X 10^6) were loaded into each lane (A and B) for electrophoretic separation. Molecular mass standards (kiloDaltons) are shown on the left side of the autoluminogram.

3.9 Analysis of mAbs 1C3 and 2G4

The monoclonal antibodies 1C3 and 2G4 were generated against Nour 6 (*EP* knock out) *T. b. brucei* PCF (Jen Chase and Morag Booy, Pearson Lab, University of Victoria, Victoria, BC.). The isotype of these two mAbs was determined to be IgG1. These mAbs were further analyzed to determine surface labeling of PCF trypanosomes by using flow cytometry and immunofluorescence microscopy. Flow cytometric analysis of live, wild type and KO1 PCF trypanosomes labeled with mAbs 1C3 and 2G4 showed that both antibodies were able to bind to antigen on live parasites **(Figure 20)**. Live wild type PCF showed 1000 fold greater fluorescence when labeled with anti-EP mAb 247 (positive control; **B**) than live KO1 PCF labeled with mAb 247 (negative control; **A**). Live, wild type and KO1 PCF trypanosomes were labeled with mAb 1C3 and showed at least 100 fold greater fluorescence than the negative controls **(C,D)**. Likewise the results for live wild type and KO1 PCF trypanosomes labeled with mAb 2G4 showed between 10-100 fold greater fluorescence than negative controls **(E,F)**. It should be noted that the intensity of fluorescence measured on wild type cells compared to KO1 PCFs was about 10 fold less, when labeled with either mAb 1C3 or mAb 1G10. Because live cells were being analyzed in these experiments the results indicated that mAb 1C3 and mAb 2G4 recognized antigens on the parasite surface.

Live and paraformaldehyde fixed wild type, KO1 and KO2 PCF trypanosomes were labeled with three different mAbs and the immunofluorescence images were captured using a CCD camera attached to an epifluorescence microscope. The different PCF trypanosomes were labeled with mAb 247 (anti-EP), mAb 1C3 (anti-Nour 6) and

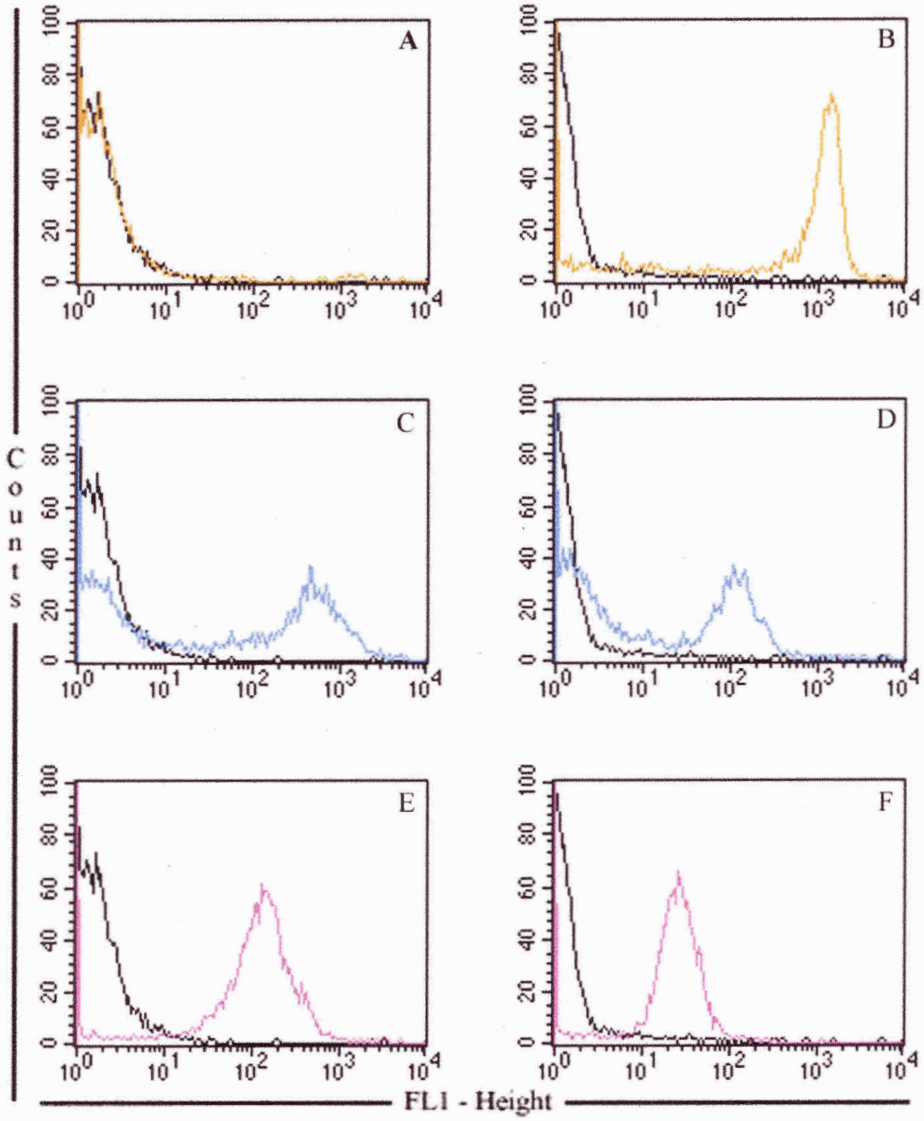

Figure 22. Flow cytometric analysis of live wild type and KO1 knock out mutant *T. b. brucei* PCF labeled with anti-Nour 6 mAbs 1C3 and 2G4. Trypanosomes (10 µL of 1 X 10^7 cells/mL) were used in each sample. The secondary antibody was Alexa Fluor 488-labeled goat anti-mouse IgG (H and L) diluted 1:400 in PBS. Panel A. Orange profile; KO1 PCF labeled with anti-EP mAb 247 (negative control). Panel B. Orange profile; wild type PCF labeled with anti-EP mAb 247 to label surface EP procyclins (positive control). Panel C. Blue profile; KO1 PCF labeled with mAb 1C3. Panel D. Blue profile; wild type PCF labeled with mAb 1C3. Panel E. Purple profile; KO1 PCF labeled with mAb 2G4. Panel F. Purple profile; wild type PCF labeled with mAb 2G4. Panels A, C, E. Black profile; KO1 PCF labeled with anti-human transferrin mAb as a negative control. Panels B, D, F. Black profile; wild type PCF labeled with anti-human transferrin mAb as a negative control. 10,000 events were recorded for each sample.

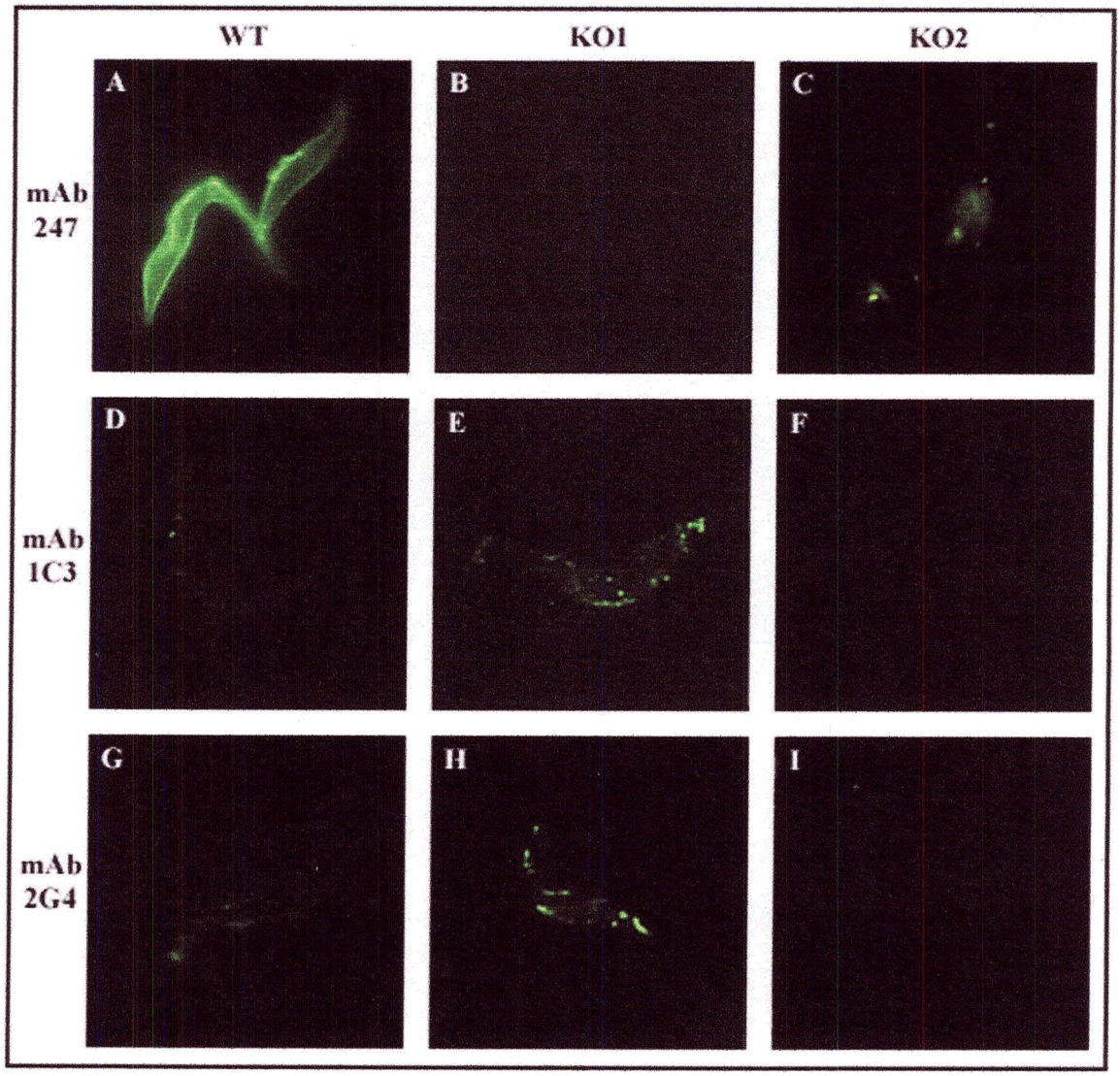

Figure 23. Immunofluorescence images of live wild type, KO1 (*EP/GPEET* knock out) and KO2 (*GPI10* knock out) *T. b. brucei* PCF labeled with anti-Nour 6 (*EP* knock out) *T. b. brucei* PCF monoclonal antibodies (mAb) 1C3 and 2G4. Live parasites were spread onto poly-L-lysine coated slides after labeling. Anti-EP mAb 247 was used to label EP procyclins on wild type PCF as a positive control on wild type parasites (A) and as a negative control on *EP/GPEET* KO1 parasites (B).

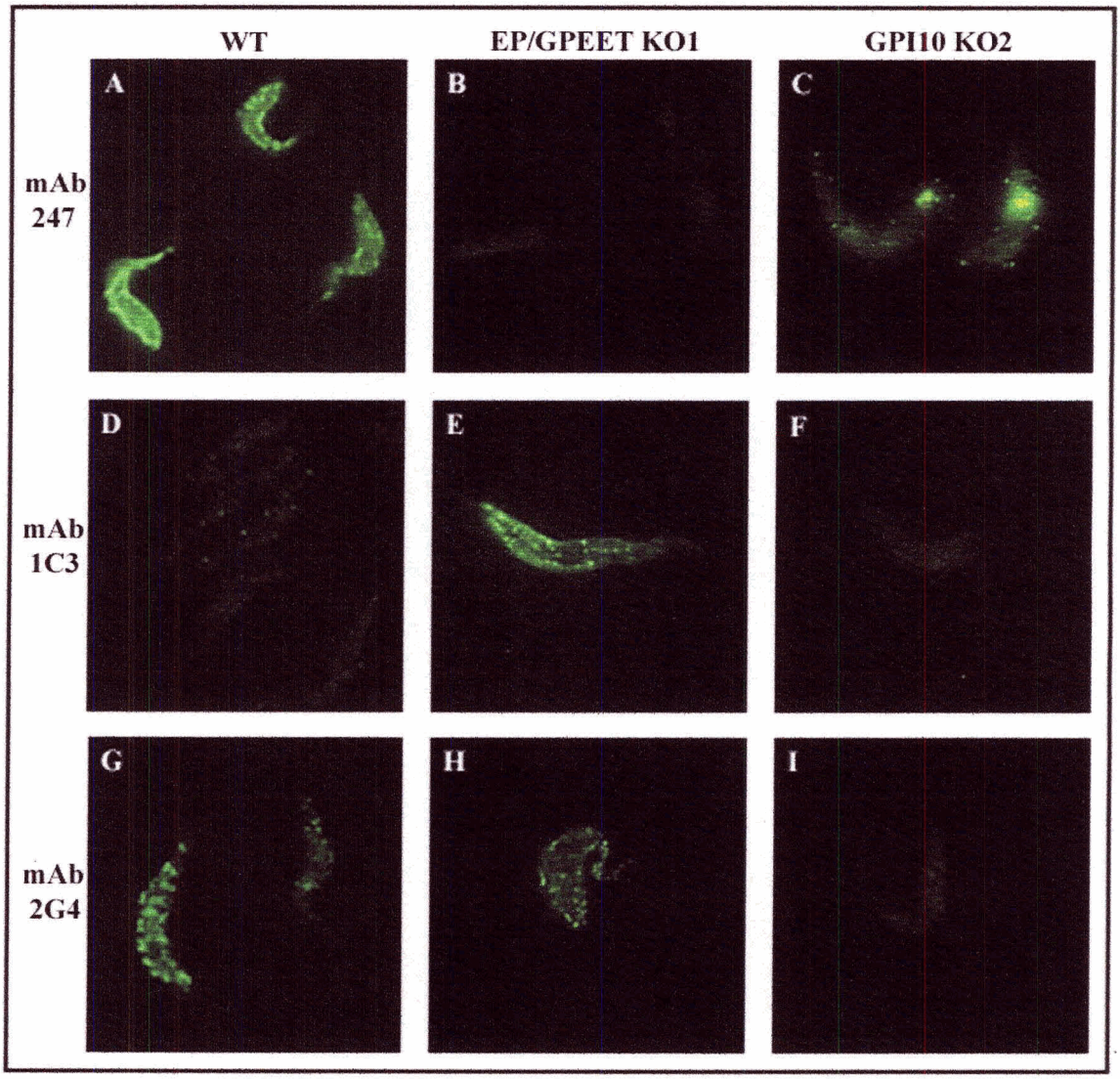

Figure 24. Immunofluorescence images of fixed wild type, KO1 (*EP/GPEET* knock out) and KO2 (*GPI10* knock out) *T. b. brucei* PCF labeled with anti-Nour 6 (*EP* knock out) *T. b. brucei* PCF monoclonal antibodies (mAb) 1C3 and 2G4. All parasites were fixed with 4% paraformaldehyde immediately prior to labeling. Anti-EP mAb 247 was used to label EP procyclins as a positive control on wild type PCF (A) and as a negative control on *EP/GPEET* KO1 PCF (B).

mAb 2G4 (anti-Nour 6) **(Figure 21;** live cells and **Figure 22;** fixed cells). The positive control used for both experiments was wild type PCF labeled with anti-EP mAb 247 and the negative control was KO1 knock out PCF labeled with mAb 247. MAb 247 labeled the entire cell surface and flagellum of both live and fixed wild type PCF **(Figure 21,22; A)** in contrast to *EP/GPEET* knock out PCF trypanosomes which showed no labeling of surface procyclins **(Figure 21,22; B)**. Live KO2 PCF trypanosomes did not show any surface labeling with mAb 247, however, there was some fluorescence in fixed KO2 PCF, mainly concentrated in one area of the cell **(Figure 21,22; C)**. This suggested that even though KO2 cells lack the necessary machinery to attach proteins to the plasma membrane with GPI anchors, they still express GPI anchored proteins, and some of this protein is retained in the cell where it can be labeled by mAb 247 if the membrane is compromised by paraformaldehyde fixation. Live wild type PCF showed very little staining with either mAb 1C3 or mAb 2G4 **(Figure 21; D,G)**. Live KO1 PCF showed increased labeling of antigens with both mAbs compared to wild type PCF **(Figure 21; E,H)**. This was similar to the flow cytometry results that showed increased fluorescence on live KO1 PCFs compared to live wild type PCFs, when labeled with mAbs 1C3 and 2G4. The staining pattern was not uniform but showed a punctate pattern on the cell surface. Live KO2 PCF were not labeled with either mAb 1C3 or mAb 2G4 **(Figure 21; F, I)**. When wild type and KO1 PCF trypanosomes were fixed with paraformaldehyde, they both exhibited more antigen labeling with mAbs 1C3 and 2G4. Fixed, wild type PCF still showed very limited amounts of labeling with mAb 1C3, but did show increased labeling with mAb 2G4 **(Figure 22; D,G)** compared to live wild type PCF. The labeling pattern was punctate, similar to that seen with live KO1 cells labeled with

either mAb 1C3 or mAb 2G4. Fixed KO1 PCF also showed increased fluorescence when labeled with mAbs 1C3 and 2G4 compared to the fluorescence observed on live cells, and the labeling pattern was punctate **(Figure 22; E,H)**. The increased fluorescence observed on fixed KO1 labeled with mAb 1C3, compared to fixed wild type, was consistent with the increased fluorescence observed on live parasites. MAb 2G4 showed similar intensities of fluorescence on fixed KO1 and wild type PCFs, even though there was no fluorescence on live wild type compared to live KO1 PCFs. Similar to the results of live KO2 parasites labeled with mAbs 1C3 and 2G4, no labeling occurred with either mAb when the parasites were fixed **(Figure 22; F,I)**. The differences in labeling of live PCF compared to fixed PCF trypanosomes indicated that the antigens recognized by mAbs 1C3 and 2G4 are less abundant than surface procyclins and are not distributed evenly over the cell surface, but appeared concentrated in small spots. The lack of labeling of both live and fixed KO2 PCF indicated that the antigens labeled with mAbs 1C3 and 2G4 were not expressed on the parasite cell surface.

In order to identify the antigens recognized by mAbs 1C3 and 2G4, 1D SDS-PAGE and electroblotting of PCF proteins was performed and the mAbs were used for immunoblotting. The results (autoluminogram) showed that neither of the mAbs recognized a protein in wild type or KO1 PCF **(Figure 23)**. This suggested that both mAb 1C3 and mAb 2G4 recognized an epitope that was destroyed upon denaturation of the protein.

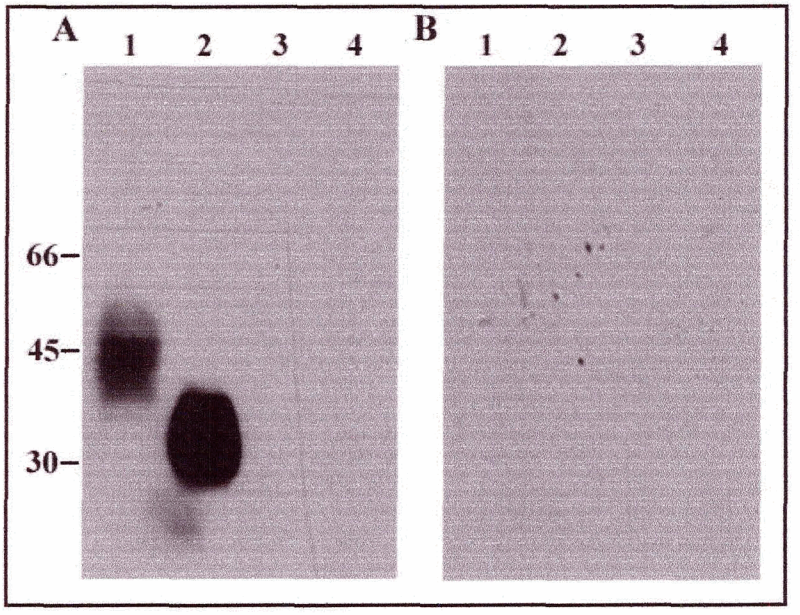

Figure 25. Immunoblot analysis of wild type and KO1 knock out mutant *T. b. brucei* PCF. Proteins of wild type (Panel A) and KO1 mutants (Panel B) using four different mAbs were separated by 1D SDS-PAGE and transferred to PVDF membrane for immunoblot analysis. Lanes 1-4 are the same for both panels. Lane 1. mAb 247 (anti-EP repeat). Lane 2. mAb 5H3 (anti-GPEET repeat). Lane 3. mAb 1C3 raised against Nour 6 (*EP* knock out) PCF. Lane 4. mAb 2G4 raised against Nour 6 (*EP* knock out) PCF. Autoluminograms were developed after 30 sec exposure. Molecular mass standards (kiloDaltons) are shown on the left side of the blot.

3.10 Analysis of biotin labeled proteins of *T. b. brucei* PCF

Biotin labeling of surface proteins with a membrane impermeable reagent was expected to detect differences in surface protein expression between the three knock out mutants, and wild type *T. b. brucei* PCF. Live PCF trypanosomes were labeled with biotin and proteins were separated by 1D SDS-PAGE and transferred to PVDF for immunoblot analysis. Autoluminograms of biotinylated proteins showed a different banding pattern for each PCF tested **(Figure 24; A)**. Biotin labeled wild type PCFs showed the expected EP and GPEET bands on the autoluminogram **(Figure 24; A)**. Wild type EP procyclins showed a smear between 40-50 kDa, and GPEET procyclins showed a smear between 25-40 kDa **(Figure 23; A)**. This strain of wild type PCFs expressed higher levels of EP procyclins than GPEET procyclins. The *EP* knock out mutant, Nour 6, showed a large, dark band between 20 and 35 kDa, indicating these mutants expressed higher levels of GPEET procyclins compared to wild type PCFs. The Nour 6 mutants do not express EP procyclins and therefore, the 40-50 kDa EP protein band was not present on the autoluminogram. Wild type PCFs showed biotin labeling of at least 4 proteins, other than the EP and GPEET procyclins. The molecular mass of these proteins was estimated from the autoluminogram using the pre-stained molecular mass markers. The proteins are roughly 55, 60, 70 and 80 kDa. These 4 protein bands also appeared in the lane containing Nour 6 PCFs however, there was an additional band present at about 50 kDa. The KO1 (*EP/GPEET* knock out) PCF trypanosomes were biotinylated most efficiently. This was observed as 14 protein bands ranging in molecular masses of about 10 kDa to 200 kDa on the autoluminogram **(Figure 24; A)**. Some of these bands overlap the region where procyclins appear.

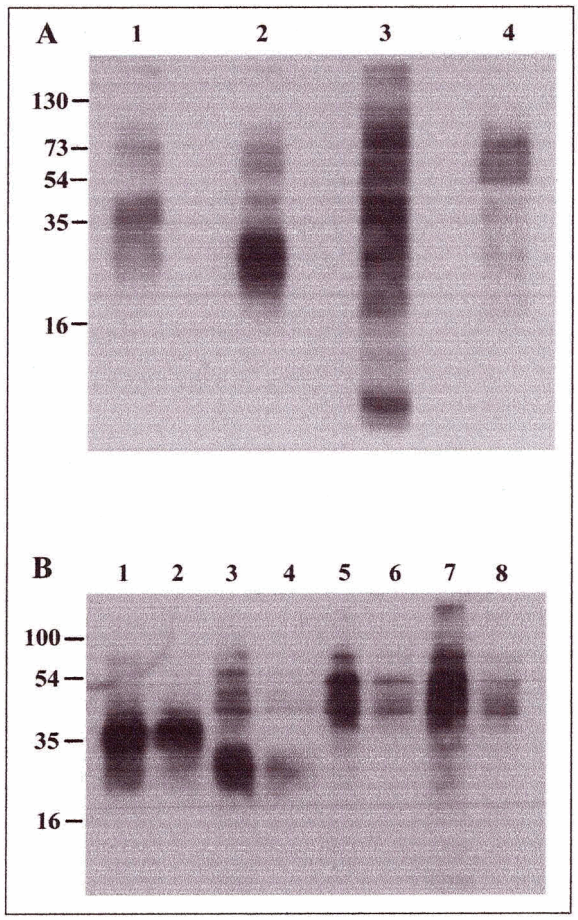

Figure 26. Blot analysis of biotinylated surface proteins of *T. b. brucei* PCF. Panel A. Detection of biotin labeled surface proteins. Trypanosomes (1 X 10^7) were labeled with Sulfo-NHS-biotin and proteins from cell lysates were separated by 1D SDS-PAGE and electroblotted onto PVDF. Streptavidin conjugated to HRPO was used to detect biotin-labeled proteins. Lane 1. Wild type 427.01 PCF. Lane 2. Nour 6 (*EP* knock out) 427.01 PCF. Lane 3. KO1 (*EP/GPEET* knock out) 427.01 PCF. Lane 4. KO2 (*GPI10* knock out) 427 PCF. Panel B. Detection of biotin-labeled proteins purified by avidin chromatography. Trypanosomes (2.5 X 10^7) were surface labeled with Sulfo-NHS-biotin, lysed and then incubated with avidin beads to bind biotin labeled proteins. Proteins bound to the avidin beads were separated by 1D SDS-PAGE and electroblotted onto PVDF. The same detection method was used as in Panel A. Lane 1 and 2. Wild type 427.01 PCF. Lane 3 and 4. Nour 6 (*EP* knock out) 427.01 PCF. Lane 5 and 6. KO1 (*EP/GPEET* knock out) 427.01 PCF. Lane 7 and 8. KO2 (*GPI10* knock out) 427 PCF. Lane 1, 3, 5, 7 were undiluted protein samples in 1X Laemmli sample buffer and Lane 2, 4, 6, 8, were diluted 1:1 in 1X Laemmli buffer. Molecular mass standards (kiloDaltons) are shown to the left of each autoluminogram.

However, pre-testing with mAbs 247 (anti-EP) and 5H3 (anti-GPEET) using immunofluorescence microscopy showed the KO1 PCFs completely devoid of any procyclins (results not shown). Therefore, there were at least 10 more proteins labeled on the surface of the KO1 mutants compared to either wild type or Nour 6 PCFs. The approximate molecular masses of these are: 10, 13, 17, 18, 20, 35, 125, 126, 150, and 200 kDa. This suggested that procyclins do impede the interaction of reacting molecules such as antibodies and biotin to less abundant surface proteins that do not protrude beyond the procyclin shield. The KO2 (*GPI10* knock out) trypanosomes showed at least 6 bands ranging from about 50 kDa to 100 kDa **(Figure 24; A)**. The approximate molecular masses are: 55, 58, 60, 65, 70, and 72 kDa. There were also two faint bands in the region where the EP and GPEET procyclin bands would be, if these proteins had been labeled with biotin. Expression of EP procyclins was observed by immunofluorescence microscopy, of live and fixed GPI10 knock out PCFs, labeled with anti-EP mAb 247 **(Figure 22,23; C),** therefore, it is known that at least EP procyclins are expressed by these mutants.

In order to identify some of the biotinylated proteins they needed to be isolated from the unlabeled proteins present in the cell lysates. Biotin labeling combined with avidin chromatography isolated different proteins from all four PCF parasites **(Figure 24; B),** similar to the banding pattern observed for biotinylated proteins without a purification step **(Figure 24; A)**. The wild type PCFs showed EP procyclins and very low levels of GPEET procyclins. The Nour 6 PCFs showed much higher levels of GPEET expression compared to wild type cells and no EP expression. The wild type and Nour 6 PCFs also showed a number of other biotin labeled proteins in a pattern similar to that seen without

the avidin chromatography **(Figure 24; A)**. Biotin labeled proteins isolated with avidin from KO1 knock out and KO2 knock out PCF trypanosomes showed at least 5 proteins ranging from 50-200 kDa on the autoluminogram **(Figure 24; B)**. The approximate molecular masses of these proteins are: 40, 42, 45, 50, 55 and 70 kDa for the KO1 knock out PCFs and 30, 40, 42, 45, 50, 55, 70 and 150 kDa for the KO2 knock out PCFs. The PVDF membranes were stained with Nigrosin after immunoblotting to verify the amount of protein transferred from the 1D gels and, to determine the efficiency of avidin to isolate biotinylated proteins from the rest of the cell lysate (results not shown). The stained PVDF membrane clearly illustrated that the amount of protein present after avidin chromatography was much less than the whole cell lysates. Avidin chromatography isolated most of the biotinylated proteins that were observed when a purification step was not used **(Figure 24; compare A and B)**. However, there was a decrease in the number of protein bands observed for the KO1 knock out PCFs.

The most significant difference in surface labeling was observed between the number of proteins labeled on the KO1 knock out PCF compared to wild type PCF **(Figure 24; A)**. Therefore, the number of KO1 knock out parasites was increased 2.5 fold (1×10^7 to 2.5×10^7), labeled with biotin, and avidin chromatography was used to isolate the labeled proteins. These proteins were separated by 2D SDS-PAGE and detected by staining with colloidal Coomassie Blue **(Figure 25)**. Over 30 different protein spots were extracted from the gel and prepared for tryptic digestion and subsequent MALDI-TOF mass spectrometry. Out of the 30 proteins analyzed, only half produced peptide mass profiles that showed tryptic peptide mass peaks such as those observed in **Figure 18**. The Protein Prospector algorithm, MS-FIT, was used to analyze

the peptide mass list obtained from the MALDI-TOF peptide mass spectrum, for each protein. From the 30 peptide mass lists analyzed, MS-FIT identified 9 proteins in the database (*Trypanosoma* / NCBI or SWISS-Pro), that produced peptide masses that matched the experimental peptide mass values. The peptide mass values in the database are generated from *in silico* fragmented proteins, to simulate trypsin digestion, and therefore, the 9 proteins were tentatively matched with proteins in the database **(Table 2)**. Three protein spots on the 2D gel (#7, #9 and #16), produced peptide mass values that were identical to peptide masses generated from α-tubulin of *T. b. rhodesiense*. Protein from spot #7 produced 32 tryptic mass peaks from the MALDI-TOF peptide mass profile, and of these, 10 had mass values that were identical to peptide mass values produced from the *in silico* fragmented α-tubulin of *T. b. rhodesiense.* This corresponded to 31% coverage, meaning that these 10 peptides constituted 31% of the whole polypeptide sequence. Details about the other protein spots, the number of peptides produced, the number of peptides matched, as well as the % coverage, have been recorded in **Appendix 1.** The peptide mass profile produced from spot #12, contained peptides with masses that matched those in the database from β-tubulin of *T. b. rhodesiense*. The spots that aligned with α-tubulin #7, #9, #16, and β-tubulin, #12, had approximate molecular masses of 70 kDa, 70, kDa, 55 kDa and 50 kDa respectively. Most of these values are larger than the molecular masses predicted by the algorithm MS-FIT (49 kDa) however 55 kDa is the estimated molecular mass given in the NCBI database for these proteins. The most acidic spot (#12) aligned with β-tubulin and the other, less acidic spots (#7, #9, #16) aligned with α-tubulin.

The proteins from spot #6 produced peptide mass values that were identical to peptide masses generated from paraflagellar rod (PFR) proteins of *T. b. brucei* **(Table 2)**. Protein from spot #6 produced 35 tryptic peptides of which 16 had mass values that matched those in the database from PFR protein. The 16 peptides constituted 26% coverage of the whole polypeptide sequence. The PFR is comprised of two major protein components, PFR 1 and PFR 2 that have a mobility range between 68 and 80 kDa. Spot #6 had approximate molecular masses of 80 kDa, again slightly higher than the predicted masses (Maga, 1999; Gull, 2001). The approximate pI value for these proteins is 6.3 and the protein spot on the 2D gel migrated to a position within this range.

Protein from spot #1 produced 11 peptides of which 8 had mass values that were identical to peptide masses from a hypothetical protein of *T. b brucei.* This protein does not show homology with any known proteins in the NCBI database. The 8 peptides that matched constitute 46% of the whole polypeptide sequence, or 46% coverage. The predicted molecular mass pf the protein was lower (85.4 kDa) than the mass indicated by the spot on the 2D gel (~100 kDa), however the approximate pI value was 7.3 and spot #1 was the most acidic spot on the gel that was matched with a protein in the database. Protein from spot #20 produced 46 peptides of which 8 had mass values that were identical to those produced from a putative coatomer β-subunit of *T. b. brucei.* These 8 peptides constituted 11% coverage of the polypeptide sequence. Protein from spot #32 produced 36 tryptic peptides of which 6 had mass values that were identical to those produced from a probable adenylate/guanylate cyclase of *T. b. brucei.* These 6 peptides constituted 7% coverage of the polypeptide sequence. The experimental peptide masses and those they matched in the database are recorded in **Appendix I.**

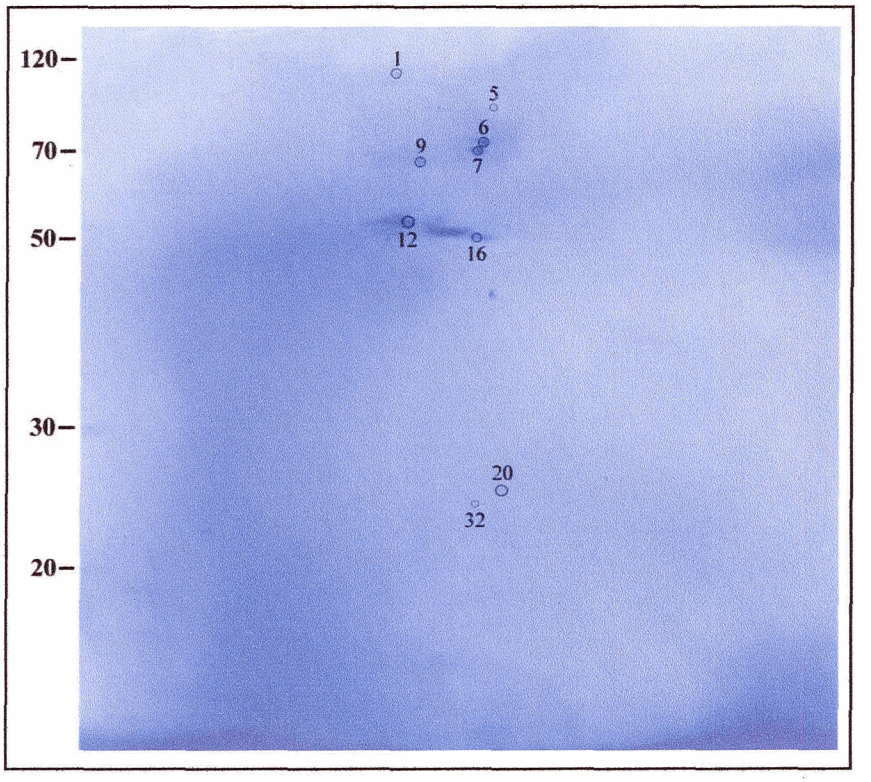

Figure 27. Two-dimensional gel separation of biotin labeled proteins isolated by streptavidin chromatography. Proteins from KO1 (*EP/GPEET* knock out) PCF trypanosomes were biotin labeled and then isolated using avidin conjugated to agarose beads. The proteins were eluted from the beads into 9M urea mix and separated by 2D SDS-PAGE. Wide range ampholines (pH 3–10) were used in the first dimension IEF, and a 10-15% gradient SDS-polyacrylamide gel for the second dimension. The proteins were detected by staining with colloidal Coomassie Brilliant Blue G-250. The protein spots extracted for trypsin digestion and MALDI-TOF mass spectrometry analysis are numbered and outlined with black circles. The gel is shown with the basic end to the right and molecular mass markers (kiloDaltons) are shown on the left side of the gel.

Table 2. Summary of proteins identified by MALDI-TOF mass spectrometry analysis of biotinylated proteins isolated by avidin chromatography and separated by 2D gel electrophoresis.

2D gel spot #	Protein ID[a] (Genbank accession No.)	MW[b] (kDa)	pI[c]	% coverage
1	*T. b. brucei* hypothetical protein (33944815)	85.4	7.3	46%
6	*T. b. brucei* paraflagellar rod protein (397891	69	6.3	26%
7	*T. b. rhodesiense* α-tubulin (2156393)	49.8	4.9	32%
9	*T. b. rhodesiense* α-tubulin (2156393)	49.8	4.9	22%
12	*T. b. rhodesiense* β-tubulin (2156392)	49.7	4.7	32%
16	*T. b. rhodesiense* α-tubulin (2156393)	49.8	4.9	28%
20	*T. b. brucei* putative coatomer β-subunit (7573281)	11	5.6	11%
32	*T. b. brucei* probable adenylate/ guanylate cyclase (Q99279)	13.8	5.8	7%

[a]Protein in the NCBInr 2005.01.06 database to which significant peptide mass matching was observed. Genbank accession numbers are provided.

[b]Predicted values determined by Protein Prospector search algorithm (MS-FIT)

[c]Predicted values determined by Protein Prospector search algorithm (MS-FIT)

The predicted molecular masses of the two proteins extracted from spot #20 and #32 were very small, 11 kDa and 13.8 kDa for coatomer and adenylate cyclase, respectively. Both of these protein spots were extracted from the lower portion of the 2D gel but migrated to positions on the gel that had an approximate molecular mass of about 25 kDa, slightly higher than the predicted. Biotinylation and avidin chromatography tentatively identified 6 different proteins from *T b. brucei* and *T. b. rhodesiense,* using MALDI-TOF mass spectrometry and the algorithm MS-FIT for peptide analysis and database matching **(Appendix I).**

5. Discussion

Culturing trypanosomes *in vitro* is not a trivial task and is a learned skill that is critical to the outcome of many experiments that depend on the use of parasite material. It is important to limit the variations in growth conditions between cultures of different forms of the parasites so that these variables do not influence the results or their interpretation. Most of the *Trypanosoma* and *Leishmania* species used in the research presented in this thesis were adapted to the same growth conditions including: medium, tissue culture flasks and temperature. The exceptions were BSF and *GPI10* knock out PCF trypanosomes. These latter two parasites have specific growth requirements and therefore require different growth conditions than the other *Trypanosoma* species (described in detail in Materials and Methods). The cultures were healthy if the parasites were very motile, no apoptotic cells were observed and when counted, over 95% were viable. The health of cultures is imperative to all experiments. For example if the parasites are undergoing apoptosis they would have compromised membranes and therefore labeling with antibodies for immunofluorescence or biotin for surface labeling would result in labeling of endogenous molecules. This creates data that compromises the experimental hypothesis and objective, and therefore it would be difficult to have confidence in the results. Consequently, the health of parasites in culture was imperative to my experiments and was given the necessary attention it deserved.

To begin a biochemical study of the surface proteins expressed by *T. b. brucei* PCF, wild type PCFs and three surface knock out PCFs were used. Protein profiles of *T. b brucei* 427.01 wild type, Nour 6 (*EP* knock out), KO1 (*EP/GPEET* knock out) and

KO2 (*GPI10* knock out) trypanosomes were analyzed using visual comparison of the protein patterns on 1D and 2D gels. The differences in proteins were expected to be most dramatic between wild type and KO2 mutants because the *GPI10* knock out mutants should lack all cell surface molecules (Nagamune *et al.*, 2000). One-dimensional gel separation did not reveal any differences in protein expression when the four different PCFs were compared. This was perhaps not surprising as 1-D separation methods are not highly resolving when complex mixtures are analyzed. However, even the more highly-resolving 2-D gels showed surprisingly few differences and these were minor. Perhaps this can be explained by the fact that the major surface molecules, the procyclins, are not easily detectable. Indeed, the discovery of procyclins was greatly hindered by the inability of these proteins to take up most kinds of protein stains (Richardson, 1988; Roditi, 1990). However, it has been shown that there are some differences between molecules expressed by the KO1, KO2 and wild type PCFs. The KO1 PCFs are sensitive to Concavalin A treatment, and express free GPI anchors on their surfaces that are sensitive to GPI-PLD activity. Concavalin A is a lectin that binds to mannose moieties found in the GPI anchors of procyclins which induces apoptosis and parasite death (Pearson *et al.*, 2000). Because of the unique lipid structure of the procyclin-GPI anchor theses molecules are insensitive to GPI-PLC cleavage but are sensitive to GPI-PLD cleavage. These characteristics have been used to distinguish procyclin-GPI anchors from regular GPI anchors that do share these characteristics. The KO2 PCFs do not express any GPI-anchored proteins on their surface, and are unable to synthesize precursor GPI anchors. Consequently these parasites do not express free GPIs on their surface. It has been reported that procyclin peptides are expressed in these parasites and

are secreted into the culture medium (Nagamune *et al.*, 2000). Knowing that these mutants express different surface molecules compared to wild type PCFs suggested that there may be other proteins that are up-regulated, or down-regulated compared to wild type. Therefore, even though the procyclins are invisible on 1D and 2D gels, it was still possible that the mutants might produce protein profiles that were different to wild type PCFs.

The next approach used to identify and characterize non-procyclin surface molecules of *T. b. brucei* PCF was performed by generating monoclonal antibodies to the non-procyclin expressing, cell surface of KO1 PCF mutants. After initial screening the mAbs 2E1 and 1G10, both IgMs, were analyzed further using flow cytometry, immunofluorescence microscopy and immunoblotting. Flow cytometry on living trypanosomes showed that mAb 1G10 did not bind antigen on the surface of either wild type or KO1 knock out PCFs. In contrast, although MAb 2E1 did not bind the surface of living KO1 PCFs, it did show 100 fold more fluorescence on wild type PCFs, compared to the negative control, indicating that the mAb was bound to the parasite. Flow cytometric analysis of the same mAbs using formaldehyde fixed trypanosomes gave different results. Both wild type and KO1 PCFs, labeled with mAbs 1G10 and 2E1, showed a level of fluorescence that was similar to the positive control of wild type PCFs labeled with anti-EP procyclin mAb, 247.

Immunofluorescence microscopy on live and paraformaldehyde fixed, wild type, KO1 and KO2 PCFs was performed to determine localization of antibody binding on the parasite. The positive controls used for both flow cytometry and immunofluorescence experiments were wild type PCFs labeled with mAb 247 (anti-EP procyclins). As

expected, live and fixed wild type cells showed strong fluorescence when labeled with mAb 247. In contrast, the KO1 PCFs (that lack both EP and GPEET procyclins) showed no fluorescence and were used as negative controls. An interesting result was the fluorescence visualized when KO2 knock out PCFs were labeled with mAb 247. These *GPI10* knock out PCFs do not express surface procyclins (Nagamune *et al.*, 2000; Lillico *et al.*, 2003). However, the immunofluorescence images do show fluorescence concentrated in one area of the parasite, indicating the presence of EP procyclins. This supports the observation by Nagamune *et al.* that immature procyclins are expressed and procyclin peptides are secreted from the cell into the culture medium. This suggests that KO2 PCFs may also express other GPI-anchored proteins and retain them within the cell, or secrete them into the culture medium. An experiment could be performed to show whether or not procyclin derivatives were present in the culture medium. Centrifugation would eliminate the parasites from the culture medium. Using a simple dot blot method procyclin peptides could be identified by immunoblot analysis with mAb 247 that would label any EP procyclins present. The most obvious problem associated with this strategy is degradation of the procyclin peptides to an extent that the 247 epitope is destroyed and can longer be recognized.

Live parasites were not labeled with mAb 1G10 however, when the cells were paraformaldehyde fixed, fluorescence was detected. MAb 1G10 strongly labeled an antigen present on all three parasites with the fluorescence pattern more intense along the sides and flagella of the parasites. Together with the lack of fluorescence on living trypanosomes, this indicated that the antigen was directly adjacent to the plasma membrane. The procyclins did not seem to play a role impeding antigen labeling on live

PCFs, because mAb 1G10 did not label antigen on the KO1 or KO2 mutants. Antigen labeling was possible after formaldehyde treatment, indicating that the fixation process somehow made the epitope recognized by mAb 1G10 accessible. Fixation can distort and change protein structure due to cross-linking therefore, a conformational change in the protein might have allowed the epitope to become exposed and labeled by mAb 1G10. Formaldehyde fixation is not known to alter cell membrane permeability, however it can not be ruled out that fixation allowed access to a protein that is integrated into the membrane structure, or is in direct contact with the membrane, so that any distortion lending to slight permeabilization would have made the antigen accessible.

MAb 2E1 bound to live wild type but not KO1 PCFs, however upon fixation it labeled wild type and KO1 PCFs and created a punctate pattern of fluorescence. These results suggest that the antigen recognized by mAb 2E1 may be a GPI-anchored protein because no fluorescence was detected on KO2 mutant PCFs. The more diffuse, punctate pattern of fluorescence indicates that the antigen might be less abundantly expressed than the antigen recognized by mAb 1G10, and definitely less abundant than the procyclins. Because procyclins were expressed in the KO2 PCFs, other GPI-anchored proteins might also be expressed in these parasites and therefore might be labeled in a similar fashion. However, the antigen recognized by mAb 2E1 was not detected on live or fixed KO2 PCFs. Procyclins are very abundant compared to other invariant surface proteins (Ziegelbauer *et al.*, 1992; Ziegelbauer *et al.*, 1992; Nolan *et al.*, 1997; Borst *et al.*, 1998; Pays *et al.*, 1998) and therefore simple concentration could account for the lack of detectable antigen with mAb 2E1. Peptides of the mAb 2E1-specific antigen could be secreted from the cell as procyclin peptides are, therefore the epitope may have been

disrupted and can no longer be recognized. It would be possible to test the culture medium for antigen presence using a dot blot method similar to the method just described for EP procyclins.

MAb 1G10 and mAb 2E1 were then tested for immunoblot reactivity on wild type and KO1 PCFs. MAb 2E1 recognized a 50 kDa protein in KO1 parasites but not wild type. This result was surprising because mAb 2E1 recognized an antigen present on both wild type and KO1 PCFs when they were formaldehyde fixed. However, when the parasites were live, mAb 2E1 only recognized an antigen present on wild type PCFs. These experiments (immunofluorescence and immunoblotting) were performed at least 10 times each and the same results were observed each time. These results were puzzling because the flow cytometry showed labeling of live wild type PCFs only, contradicting the immunoblot which showed labeling of KO1 PCFs only. However, when the parasites were fixed, immunofluorescence showed labeling on both wild type and KO1 PCFs. The discrepancy between the flow cytometry of live parasites and the immunofluorescence of fixed parasites could possibly be explained by the presence (wild type) or absence (KO1) of procyclins. It is possible that the density of procyclins on the surface excludes or precludes other, less abundant surface proteins that might occupy the surface when procyclins are not present. An isotype control was used, however it can not be ruled out that the labeling seen on live wild type PCFs could be due to non-specific binding of the mAb to a surface protein. However the mAb is actually specific for a protein present on KO1 PCFs which becomes accessible after fixation and mAb 2E1 binds to produce to punctate fluorescence pattern. To control for non-specific binding a good experiment would be to titre the antibody using fluorescence on fixed wild type and KO1 PCFs. If

mAb 2E1 is binding non-specifically to something on wild type PCFs fluorescence of the KO1 PCFs will titre out to much lower concentrations of mAb while labeling of the wild type will disappear quickly. It is possible that mAb 2E1 does recognize an antigen expressed on both wild type and KO1 PCFs. It is known that the KO1 mutant expresses four times more GPI anchors on its surface compared to wild type (Nagamune *et al.*, 2000). It is possible that these parasites increase expression of other surface molecules in compensation for the lack of procyclins. The antigen recognized by mAb 2E1 may be present in larger numbers on KO1 PCFs compared to wild type PCFs. Consequently, when immunoblotting with mAb 2E1 there is enough protein present to be labeled on KO1 but not wild type PCFs. This could be tested by loading increasing numbers of wild type PCFs onto a 1D gel to see if the protein band at 50 kDa appears once the amount of protein present reaches the necessary threshold. Immunofluorescence is more sensitive than immunoblotting which would explain why antigen labeling could be seen on wild type PCFs.

MAb 1G10 recognizes a 55 kDa protein on 1D immunoblots of wild type and KO1 PCFs. The labeled protein appears to be abundant because it produces a very dark, large band on the autoluminogram after only a one second exposure time of the film to the immunoblot. This is consistent with the very bright fluorescence observed when fixed PCFs were labeled with mAb 1G10. The intensity of fluorescence was similar to that observed when EP procyclins are labeled on wild type PCFs.

Two-dimensional gel electrophoresis has greater resolving power than 1D gel electrophoresis because two different, unrelated parameters (isoelectric point and charge/mass ratio) are used to separate complex protein mixtures. Proteins of the same

molecular weight that appear as one band on 1D gels might have different isoeletric points (pI) and thus would most likely appear as individual spots on 2D gels. Two-dimensional immunoblotting with mAb 2E1 was unsuccessful and multiple attempts only yielded empty autoluminograms. The electrophoretic transfers were working as verified by staining of PVDF membranes with Nigrosin. This suggests that the antigen recognized by mAb 2E1 undergoes a conformational change when solubilized in the highly denaturing environment (greater than 1X Laemmli for SDS-PAGE) of 9M urea for 2D gel electrophoresis. This alters the epitope and mAb 2E1 can no longer recognize and bind to the protein. The lack of 2D immunoblot activity could be due to a simple abundance issue. Using two dimensions to separate protein mixtures usually requires 10 times more protein than 1D gels. Using different numbers of parasites was tried in attempts to reach the threshold amount for immunoblotting with mAb 2E1. Parasite numbers were increased 2 fold, 5 fold and 10 fold, the usual number (1×10^7). The antigen may also be very acidic or basic and therefore would be difficult to resolve on a 2D gel. Very basic proteins have difficulty entering the first dimension, and both acidic and basic ends of the second dimension always seem to give poor resolution, showing vertical smears especially when the gel is overloaded with too much protein. Immunoblotting with mAb 2E1 was unsuccessful.

On the other hand, 2D immunoblotting with mAb 1G10 worked very well on both wild type and KO1 PCFs. The autoluminograms showed a smear at 55 kDa similar to the molecular weight of the protein recognized by mAb 1G10 on 1D immunoblots. The next step was to isolate the protein(s) recognized by mAb 1G10 on a 2D gel stained with colloidal Coomassie Blue. Nine protein spots were cored and analyzed using MALDI-

TOF mass spectrometry and the algorithm MS-FIT to identify the protein(s) **(Appendix I)**. All nine spots produced peptides with mass values identical to those produced by *in silico* fragmentation of tubulin from *T. b. rhodesiense.* Analysis of the peptide mass profiles for each spot produced interesting results. Depending on where in the smear of protein the 2D gel spot was extracted, the peptide masses aligned with β- or α-tubulin exclusively, or with both. The predicted molecular masses for both proteins were virtually the same (49 kDa). However the predicted pI values were slightly different, 4.7 for β-tubulin and 4.9 for α-tubulin. Given these predicted pI values the results produced by MS-FIT were as expected. The two most acidic spots produced peptides with mass values that matched those of peptides from β-tubulin, and all the rest, less acidic spots, produced peptides with mass values that matched peptides from α-tubulin. One spot, # 5, did show peptides with masses that matched both α-tubulin and β-tubulin, although the match with α-tubulin was stronger. The number of peptides matched with α-tubulin was 9, and 5 peptides matched with β-tubulin. It should be noted that the peptides that matched one of these proteins were not the same that matched the other. This infers that there was both tubulin forms present in the protein spot cored for this sample. This does not infer that mAb 1G10 binds to both forms of tubulin as these two proteins only share 40% polypeptide sequence identity. Although mAb 1G10 creates a smear on an immunoblot that overlaps the area containing both tubulin proteins on the 2D gel, it is the MALDI-TOF mass spectrometry and the protein algorithm, MS-FIT, used to analyze the peptide mass profiles that detect the form of tubulin present in a spot. Since α-tubulin and β-tubulin only share 40% identity it is unlikely, but not impossible, that mAb 1G10 recognizes and binds to both proteins. The peptide sequences that matched peptides in

the database generated from β-tubulin are not the same peptides that matched peptides generated from α-tubulin. It is possible that mAb 1G10 is specific for α-tubulin however; in spot #1 and #2 the majority of protein present is β-tubulin. Therefore, mAb 1G10 bound to the small amount of α-tubulin present in the spot, but MS-FIT identified the protein as β-tubulin because most of the peptides have mass values that matched β-tubulin peptides in the database. The possibility that mAb 1G10 does bind a common epitope in both tubulin proteins can not be ruled out.

To verify the mass spectrometry results that identify tubulin as the antigen recognized by mAb 1G10, further experiments could be done. Tubulin could be isolated and purified from trypanosomes and the ability of mAb 1G10 to bind this protein could be tested by indirect ELISA. To be more specific, the trypanosome genes encoding β-tubulin and α-tubulin could be cloned into a plasmid and expressed in a mouse or human cell line. The cell line would need to be pre-tested with mAb 1G10 by immunoblotting and immunofluorescence, and be negative for both. These cells would not express any other trypanosome proteins and therefore if mAb 1G10 recognized a protein on an immunoblot, it would further demonstrate that this mAb is specific for trypanosome tubulin. It might also determine what form of tubulin mAb 1G10 recognizes because β-tubulin and α-tubulin could be expressed separately and mAb 1G10 may react with one or the other, or both.

MAb 1G10 became increasingly intriguing when its specificity was determined by immunoblotting on proteins from different *Trypanosoma* species, *Leishmania* and mammalian cells. MAb 1G10 recognizes a 55 kDa protein in both PCF and BSF trypanosomes and was therefore not stage specific. This was expected as there are no

differences between α-tubulin or β-tubulin polypeptide sequences expressed by PCF and BSF. There are very minor differences between *T. b. brucei* tubulins and the tubulins expressed by *T. simiae* and *T. congolense.* There are more differences in the protein sequences of *Leishmania* tubulins (93% identical to *T. b. brucei*) and especially mouse and human tubulins (84% identical to *T. b. brucei*); therefore it was of great interest to determine whether or not mAb 1G10 would recognize the 55 kDa tubulin protein in other kinetoplastids and mammalian cells. Given that tubulin is one of the most evolutionarily conserved proteins in eukaryotes it was surprising to find that mAb 1G10 recognized tubulin in other *Trypanosoma* species and *Leishmania* species but did not bind to tubulin expressed by either the mouse or human cells tested. This indicates that mAb 1G10 may be specific for tubulin expressed by kinetoplastids. It has recently been shown that immunization with a crude extract of trypanosome tubulin can protect mice from a lethal dose of *T. b. rhodesiense* (Lubega *et al.*, 2002; Lubega *et al.*, 2002; Ochola *et al.*, 2002). In contrast, tubulin extracted from rat brain did not induce immunity against trypanosome challenge (Lubega *et al.*, 2002; Lubega *et al.*, 2002; Ochola *et al.*, 2002). This data indicates there are significant differences between trypanosome and mammalian tubulin because the mice did not develop autoimmunity to self expressed tubulin and it was only trypanosome tubulin that conferred resistance. Sequence identity between trypanosome tubulin and mouse and human tubulin is only 83%, in contrast to the 100% identity shared between mouse and human tubulin sequences. The ability to immunize mice with trypanosome tubulin and the fact that mAbs can be generated to recognize trypanosome tubulin but not mammalian tubulin supports the hypothesis that there are B-cell epitopes presented by trypanosome tubulin and not mammalian tubulin. Tubulin is a very

abundant protein in trypanosomes and has multiple functions including: 1) providing the parasite with flexibility, mechanical support and mobility as microtubules that comprise the cytoskeleton, 2) forming the axoneme, one of the two predominant components of the flagellum and 3) functioning in the mitotic spindle apparatus of dividing nuclei (Lubega *et al.*, 2002). Given that tubulin is an intracellular protein, how does immunization with trypanosome tubulin induce immunity? In BSF trypanosomes, VSGs form a dense coat over the entire cell surface except the flagellar pocket, the only site of endocytosis and exocytosis. The VSG coat has a very high turnover rate and extensive vesicular trafficking occurs via the flagellar pocket. Therefore tubulin antibodies could potentially enter the flagellar pocket and be internalized by the parasite via endocytosis. If the antibody were released into the cytoplasm it could bind microtubules essential for vital parasite functions such as motility and division, thereby causing parasite death. This would be an interesting experiment to do in the future. Lubega *et al.* also showed that anti-trypanosome tubulin mAbs induce parasite death *in vitro*. It would be interesting to repeat this experiment with mAb 1G10 to see if it has trypanocidal activity. If mAb 1G10 is capable of inducing parasite death, it would be imperative to determine whether or not the mAb is internalized by the parasite. Looking at the parasites incubated with purified mAb 1G10 over a time course using scanning confocal fluorescent microscopy might enable mAb localization in the parasite. If the mAb is internalized this information would give insight into the mechanism by which it exhibits its trypanocidal activity.

The other monoclonal antibodies (1C3 and 2G4) analyzed in this research were previously generated against Nour 6 (*EP* knock out) PCF *T. b. brucei* by Jen Chase and Morag Booy in the Pearson lab. To further characterize these mAbs, the surface knock

out mutants of PCF trypanosomes were employed in flow cytometric, immunofluorescence and immunoblot assays. Using flow cytometry these mAbs showed labeling of live wild type and KO1 PCFs indicating that they might recognize surface antigens. Immunofluorescence on both live and fixed wild type, KO1 and KO2 PCFs was used to characterize them further. On living parasites mAb 1C3 and 2G4 labeled the KO1 PCF and only showed very small amounts of labeling on the wild type PCFs. Immunofluorescence on fixed parasites resulted in increased labeling of wild type PCF when compared to the labeling observed with live PCFs. Both live and fixed KO2 PCFs showed no labeling with either mAb 1C3 or mAb 2G4. This lack of labeling on the GPI10 knock out PCFs implies that the antigens recognized by these mAbs could be surface proteins. This is supported by the observation of the same immunofluorescence pattern on live and fixed KO1 PCFs, indicating that the mAbs recognize a native epitope on the surface of living PCFs. The increased fluorescence seen when wild type PCFs were fixed compared to live indicates that the surface procyclins interfere with the binding of mAbs to the underlying surface antigens they recognize. The fluorescence pattern is also distinctive in that it is not uniform but rather punctate over the entire cell surface, suggesting that the abundance of these molecules is less than that of the surface procyclins. All of these results are not unexpected as it has previously been reported numerous times that procyclins shield the underlying membrane surface (Ziegelbauer *et al.*, 1992; Ziegelbauer *et al.*, 1992; Nolan *et al.*, 1997; Borst *et al.*, 1998; Pays *et al.*, 1998). Protecting underlying membrane proteins is how procyclins function as a shield against proteases and other immune molecules in the tsetse fly vector as has been suggested (Roditi and Pearson, 1990). The procyclins are also the most abundant surface

proteins on PCFs and therefore a more diffuse, fluorescence pattern would be expected of less abundant, underlying surface proteins. The use of different surface knock out mutants of PCFs was a very effective approach to further characterize the anti-KO1 mAbs and gain some insight into the antigens they recognize.

MAbs 1C3 and 2G4 were analyzed for immunoblot reactivity and both were unable to recognize a protein in wild type or KO1 cell lysates. Therefore, the 2D immunoblot and mass spectrometry approach used to identify the tubulin antigen recognized by mAb 1G10 could not be applied to these mAbs. Numerous attempts were made to isolate the antigens by immunoaffinity chromatography. Different procedures were tried including the use of different buffers, different detergents to solubilize the antigens out of the membrane, and different solid matrices to bind the antigen-antibody complexes. These attempts were futile and this line of research came to a stop.

The final approach used to identify novel cell surface proteins on *T. b. brucei* PCF was surface biotinylation of three different surface mutants of PCF trypanosomes. Sulfo-NHS-biotin is a membrane impermeable reagent and thus useful for surface labeling of healthy parasites. However, if the parasites are not in log phase growth or there are any compromised cells present in the culture then the biotin reagent gains entry into the cells and can label a multiplicity of proteins. Biotinylation and immunoblotting with streptavidin conjugated to HRPO was used to identify surface proteins expressed by wild type, Nour 6, KO1 and KO2 PCFs. Biotinylation of these four PCFs was expected to yield different protein banding patterns for each parasite, given the fact that they express different surface molecules due to the various knock out mutations they contain. The wild type PCFs, which express both forms of procyclins and the underlying dense GPI

glycocalyx, were expected to produce protein bands typical of EP and GPEET procyclins when biotinylated. Access to underlying, less abundant surface molecules would most likely be hidden by the procyclin shield and unlikely to be labeled with biotin. In contrast, Nour 6 parasites that only express GPEET procyclins, should lack the protein band typical of the EP procyclins, and might show other labeled proteins due to a more diffuse procyclin shield. The most striking differences were expected to be observed for proteins labeled on the KO1 and KO2 PCFs due to the complete lack of all procyclins on both of these knock out mutants, and the lack of all GPI anchored proteins on the KO2 knock out. Without the procyclins the procyclin-negative cell surface of PCF trypanosomes would be exposed and underlying, less abundant molecules could potentially be labeled with biotin. The KO2 PCFs might show very few surface labeled proteins because there might be very few membrane proteins that are not GPI-anchored. The KO1 PCFs showed the greatest number of biotinylated proteins with at least 10 more proteins labeled compared to the wild type and Nour 6 PCFs. This indicates that procyclins do inhibit the interaction of reacting molecules such as antibodies and biotin to less abundant surface proteins that do not protrude beyond the procyclin shield. Even though the *GPI10* knock out PCFs lack the machinery to attach GPI anchors to proteins destined for the plasma membrane, they still produce these proteins as observed with immunofluorescence of labeled procyclins when using the mAb 247 (anti-EP). Knowing that these KO2 PCFs still produce GPI-anchored proteins, such as the very abundant procyclins, it was important that no procyclins be biotinylated on these cells. However, there were very faint bands on the streptavidin blot where EP and GPEET procyclins would usually be, indicating the membrane may have been compromised in some of the

parasites labeled. A good control to run in parallel with this experiment would have been to purposely permeabilize the membrane of all four PCFs and then label the parasites with biotin. This would have shown the difference in proteins labeled on live healthy parasites with supposedly impermeable membranes to those parasites that definitely had permeable membranes.

Avidin conjugated to agarose beads was successful in isolating most of the biotinylated proteins labeled on the original blot of biotinylated proteins in whole cell lysates. Therefore, this approach was used to isolate biotinylated surface antigens from KO1 PCFs for separation on 2D gels and MALDI-TOF mass spectrometry. More than 30 different spots were extracted from the 2D gel; however, many spots were extremely faint even when incubated in colloidal Coomassie Blue stain for more than one week. Thus it is not surprising that only 15 of the protein spots generated peptide mass profiles with enough tryptic peptides to search for mass matches. From these 15 spots, 9 showed high scores with proteins in the NCBI database when searching trypanosome sequences. Protein spot #1 showed sequence alignment to a hypothetical protein that does not show similarity to any known protein in the NCBI database. However, most of the protein spots with good peptide mass profiles showed significant matches to either tubulin or the paraflagellar rod protein. On BSF trypanosomes, VSG supposedly covers the entire parasite surface except the flagellar pocket. However, on PCF trypanosomes the procyclins form a more diffuse coat around the parasite and proteins such as tubulin and the paraflagellar rod protein may be more exposed. These two proteins comprise the flagellum (Wrightsman *et al.*, 1995; Maga, 1999; Gull, 2001; Lubega *et al.*, 2002; Lubega *et al.*, 2002; Ochola *et al.*, 2002), and therefore might be surface exposed on the

portion of the flagellum that is not attached to the parasite membrane, thus biotin might be able to label these proteins at this interface. It is also possible that a small population of parasites had semi-permeable membranes, even though parasites were analyzed visually and counts were done to ensure greater than 98% viability just prior the labeling experiment. However, if a few parasites had compromised membrane then biotin would have been able to label at least the two most abundant proteins that are also in very close, if not direct, contact with the plasma membrane (tubulin and PFR proteins).

Protein spot #20 produced peptides with masses that matched those of a putative coatomer protein. This is a multisubunit complex involved in trafficking of vesicles between the endoplasmic reticulum and the Golgi apparatus (Maier, 2001). The flagellar pocket is the only area where endocytosis and exocytosis occurs in BSF trypanosomes and extensive vesicular trafficking is seen near the Golgi apparatus (Clayton, 1995; Bangs, 1998; Maga, 1999). It is hypothesized that this is also true of PCF trypanosomes, although it occurs at a much lower rate. Therefore, it is not completely improbable that the proteins found in vesicles are exposed to the parasite surface at the flagellar pocket interface where molecules such as biotin could enter and bind to proteins present. The other protein identified by surface biotinylation (spot #32) was a probable adenylate/guanylate cyclase. This transmembrane protein is known to have a receptor-like structure that has been localized to the plasma membrane and flagellar pocket of BSF trypanosomes (Borst *et al.*, 1998; Pays *et al.*, 1998). Thus it is possible that PCF trypanosomes also express these receptors as they play an integral role in cellular response to extracellular signals (Kelly, 2004). These results imply that the naked (no procyclins) KO1 surface exposed otherwise hidden, less abundant surface molecules to

be labeled and identified using biotinylation, avidin purification and MALDI-TOF mass spectrometry.

The objective of this research was to use procyclin knock out mutants of *T. b. brucei* PCF to identify less abundant surface molecules. Attempting to analyze the total protein profiles of four different PCFs was unsuccessful due to the complexity of trypanosome protein lysates and the inability to visualize procyclins on SDS-polyacrylamide gels. Using monoclonal antibodies generated against two different procyclin knock out PCFs was more successful. However, the only antigen identified in this way was the abundant α- and β-tubulins expressed by *T. b. rhodesiense*. The ability of a mAb to recognize the specific epitope on immunoblots was a great asset for identifying the antigen recognized by mAb 1G10. Other immunological methods implied that the antigens recognized by mAbs 2E1, 1C3 and 2G4 are most likely surface proteins although further characterization was unsuccessful. Knowing that these antigens are could be beneficial for their isolation in the future. Immunoprecipitation with the antigen-specific mAbs and NP-40 solubilization could possibly work to isolate the antigen-antibody complex. Cleaving off the GPI anchor with GPI-PLC creates a new epitope termed the cross reacting determinant (CRD) that can be recognized by immunoblotting with anti-CRD mAb. This experiment offers a method not tried in this research, but one that may help isolate and identify the unknown antigens recognized by mAbs 2E1, 1C3 and 2G4.

Surface biotinylation proved to be an effective method for isolating surface antigens expressed by procyclin knock out PCF trypanosomes. In the future more focus should be given to experiments using different biotinylation reagents and increased

numbers of parasites to try and isolate more surface proteins for identification. Biotin reagents such as biotin-LC-hydrazide could be used to label and isolate surface exposed carbohydrates that might be linked to membrane proteins that are otherwise shielded from labeling reagents. Characterization of biotin labeled molecules on the surface of the *GPI10* knock out might prove interesting since these molecules might be transmembrane proteins with receptor or transporter functions essential for parasite viability.

The use of different procyclin knock out mutants of PCF *T. b. brucei* was essential to the success of this research. The *EP/GPEET* knock out PCF allowed access to underlying, less abundant surface proteins that are otherwise invisible to monoclonal antibodies and other labeling reagents such as biotin. The *GPI10* knock out PCF allowed further characterization of unknown antigens recognized by procyclin knock out specific mAbs.

Although it would be ideal to study surface antigens expressed by BSF trypanosomes, using procyclin knock out PCF trypanosomes might prove invaluable in identifying new surface exposed molecules that might also be expressed by BSF. The resurgence of African sleeping sickness and the increase in drug resistance has created a desperate need for vaccines and chemotherapeutic targets. Studying surface molecules on PCF mutants might turn out to be the key to identifying a novel target for the elusive vaccine.

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Appendix I

A. MS-FIT analysis of peptide mass lists from proteins extracted from a 2D gel corresponding to the proteins recognized by mAb 1G10.

i. Spot #1

MOWSE Score	#/44(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
2509	**10 (22)**	**20.0**	**-93.7**	**43.0**	**49705/4.7**	**P04107**	TRYBR	Tubulin beta chain (Beta tubulin)

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1041.4604	1041.5733	-108		310	318	0	(R) YLTASALFR (G)
1076.4315	1076.5489	-109		155	162	1	(K) LREQYPDR (I)
1125.5599	1125.6784	-105		253	262	0	(K) LAVNLVPFPR (L)
1146.4715	1146.5907	-104		242	251	0	(R) FPGQLNSDLR (K)
1243.4804	1243.6145	-108	1Met-ox	381	390	0	(R) VGEQFTLMFR (R)
1253.6506	1253.7734	-98		252	262	1	(R) KLAVNLVPFPR (L)
1274.5589	1274.6857	-100		242	252	1	(R) FPGQLNSDLRK (L)
1341.5138	1341.6439	-97		47	58	0	(R) INVYFDEATGGR (Y)
1383.5956	1383.6374	-30		298	309	1	(K) NMMQAADPRHGR (Y)
1383.5956	1383.7207	-90		380	390	1	(R) RVGEQFTLMFR (R)
1383.5956	1383.7207	-90		381	391	1	(R) VGEQFTLMFRR (K)
1647.6873	1647.8264	-84	1Met-ox	63	77	0	(R) SVLIDLEPGTMDSVR (A)

ii. Spot # 2

MOWSE Score	#/36(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
1.102e+06	**11 (30)**	**30.6**	**37.3**	**128834**	**49705/4.7**	**P04107**	TRYBR	Tubulin beta chain (Beta tubulin)

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1076.5627	1076.5489	13		155	162	1	(K) LREQYPDR (I)
1125.6952	1125.6784	15		253	262	0	(K) LAVNLVPFPR (L)
1146.6076	1146.5907	15		242	251	0	(R) FPGQLNSDLR (K)
1243.6410	1243.6145	21	1Met-ox	381	390	0	(R) VGEQFTLMFR (R)
1253.8006	1253.7734	22		252	262	1	(R) KLAVNLVPFPR (L)
1274.7076	1274.6857	17		242	252	1	(R) FPGQLNSDLRK (L)
1341.6745	1341.6439	23		47	58	0	(R) INVYFDEATGGR (Y)
1383.7797	1383.6374	103		298	309	1	(K) NMMQAADPRHGR (Y)
1383.7797	1383.7207	43		380	390	1	(R) RVGEQFTLMFR (R)
1383.7797	1383.7207	43		381	391	1	(R) VGEQFTLMFRR (K)
1399.7676	1399.6323	97	1Met-ox	298	309	1	(K) NMMQAADPRHGR (Y)
1399.7676	1399.7156	37	1Met-ox	380	390	1	(R) RVGEQFTLMFR (R)
1399.7676	1399.7156	37	1Met-ox	381	391	1	(R) VGEQFTLMFRR (K)
1679.9075	1679.8314	45	1Met-ox	283	297	0	(R) GLSVPELTQQMFDAK (N)
1724.9170	1724.8648	30		337	350	0	(K) NSSYFIEWIPNNIK (S)

iii. Spot # 3

MOWSE Score	#/32(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
1.543e+10	**11 (34)**	**34.0**	**-1.75**	**39.8**	**49788/4.9**	**P04106**	TRYBR	TUBULIN ALPHA CHAIN

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1132.5275	1132.5672	-35		113	121	0	(K) EIVDLCLDR (I)
1396.7275	1396.6935	24		391	401	1	(R) IDHKFDLMYSK (R)
1396.7275	1396.7589	-22		85	96	0	(R) QLFHPEQLISGK (E)
1412.6767	1412.6884	-8.3	1Met-ox	391	401	1	(R) IDHKFDLMYSK (R)
1443.8254	1443.8535	-19		230	243	0	(R) LIGQVVSSLTASLR (F)
1525.7676	1525.7691	-0.96		340	352	0	(R) TIQFVDWSPTGFK (C)
1701.8877	1701.9063	-11		65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.8795	1718.8826	-1.8		216	229	0	(R) NLDIERPTYTNLNR (L)
1774.9653	1774.9743	-5.1		265	280	0	(R) IHFVLTSYAPVISAEK (A)
2330.0321	2330.0188	5.7		403	422	0	(R) AFVHWYVGEGMEEGEF SEAR (E)

2401.2437	2401.1900	22	85	105	1	(R) QLFHPEQLISGKEDAAN NYAR (G)
2409.2828	2409.2091	31	244	264	0	(R) FDGALNVDLTEFQTNLV PYPR (I)

iv. Spot # 4

MOWSE Score	#/48(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
2.335e+05	**7 (14)**	**16.0**	**-52.4**	**33.5**	**49788/4.9**	**P04106**	TRYBR	TUBULIN ALPHA CHAIN

m/z Submitted	MH$^+$ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1132.4875	1132.5672	-70		113	121	0	(K) EIVDLCLDR (I)
1396.6670	1396.6935	-19		391	401	1	(R) IDHKFDLMYSK (R)
1396.6670	1396.7589	-66		85	96	0	(R) QLFHPEQLISGK (E)
1412.6046	1412.6884	-59	1Met-ox	391	401	1	(R) IDHKFDLMYSK (R)
1525.6903	1525.7691	-52		340	352	0	(R) TIQFVDWSPTGFK (C)
1701.8061	1701.9063	-59		65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.7847	1718.8826	-57		216	229	0	(R) NLDIERPTYTNLNR (L)
1874.9137	1874.9837	-37		215	229	1	(R) RNLDIERPTYTNLNR (L)

v. Spot # 5

MOWSE Score	#/48(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
1.237e+06	**9 (18)**	**32.0**	**-47.0**	**28.5**	**49788/4.9**	**P04106**	TRYBR	TUBULIN ALPHA CHAIN
719	**5 (10)**	**19.0**	**-76.3**	**40.2**	**49573/4.7**	**P08562**	TRYCR	Tubulin beta chain (Beta tubulin)

Acc. #: P04106 **Species:** TRYBR **Name:** TUBULIN ALPHA CHAIN

m/z Submitted	MH$^+$ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1396.6579	1396.6935	-26		391	401	1	(R) IDHKFDLMYSK (R)

1396.6579	1396.7589	-72	85	96	0	(R) QLFHPEQLISGK (E)
1443.7677	1443.8535	-59	230	243	0	(R) LIGQVVSSLTASLR (F)
1525.6809	1525.7691	-58	340	352	0	(R) TIQFVDWSPTGFK (C)
1701.8208	1701.9063	-50	65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.8170	1718.8826	-38	216	229	0	(R) NLDIERPTYTNLNR (L)
1774.8834	1774.9743	-51	265	280	0	(R) IHFVLTSYAPVISAEK (A)
2345.9387	2346.0137	-32 1Met-ox	403	422	0	(R) AFVHWYVGEGMEEGEF SEAR (E)
2401.0778	2401.1900	-47	85	105	1	(R) QLFHPEQLISGKEDAA NNYAR (G)
2409.1220	2409.2091	-36	244	264	0	(R) FDGALNVDLTEFQTNL VPYPR (I)

Acc. #: P08562 **Species:** TRYCR **Name:** Tubulin beta chain (Beta tubulin)

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1146.5122	1146.5907	-68		242	251	0	(R) FPGQLNSDLR (K)
1396.6579	1396.7220	-46	2Met-ox	163	174	0	(R) IMMTFSIIPSPK (V)
2315.9380	2316.1303	-83		360	379	1	(K) GLKMAVTFVGNNTCIQE MFR (R)
2359.9416	2360.1432	-85		263	282	1	(R) LHFFMMGFAPLSSRGSQ QYR (G)
2717.1070	2717.3755	-99		217	241	0	(K) LTTPTFGDLNHLVSAVVS GVTCCLR (F)

vi. Spot # 6

MOWSE Score	#/48(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
1.575e+05	**9 (18)**	**26.0**	**-42.4**	**45.5**	**49788/4.9**	**P04106**	TRYBR	TUBULIN ALPHA CHAIN

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
888.4267	888.4678	-46		423	430	0	(R) EDLAALEK (D)
909.4279	909.5046	-84		157	164	1	(R) LSVDYGKK (S)
1132.5425	1132.5672	-22		113	121	0	(K) EIVDLCLDR (I)

1396.6848	1396.6935	-6.2	391	401	1	(R) IDHKFDLMYSK (R)
1396.6848	1396.7589	-53	85	96	0	(R) QLFHPEQLISGK (E)
1443.7865	1443.8535	-46	230	243	0	(R) LIGQVVSSLTASLR (F)
1525.6985	1525.7691	-46	340	352	0	(R) TIQFVDWSPTGFK (C)
1701.8567	1701.9063	-29	65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.8395	1718.8826	-25	216	229	0	(R) NLDIERPTYTNLNR (L)
1862.7207	1862.8426	-65	312	326	1	(K) YMACCLMYRGDVVPK (D)

vii. Spot # 7

MOWSE Score	#/52(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
3.015e+06	**11 (21)**	**34.0**	**-7.45**	**55.9**	**49788/4.9**	**P04106**	TRYBR	TUBULIN ALPHA CHAIN

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1396.7129	1396.6935	14		391	401	1	(R) IDHKFDLMYSK (R)
1396.7129	1396.7589	-33		85	96	0	(R) QLFHPEQLISGK (E)
1401.6593	1401.7524	-66		113	123	1	(K) EIVDLCLDRIR (K)
1412.6655	1412.6884	-16	1Met-ox	391	401	1	(R) IDHKFDLMYSK(R)
1443.8346	1443.8535	-13		230	243	0	(R) LIGQVVSSLTASLR (F)
1525.7450	1525.7691	-16		340	352	0	(R) TIQFVDWSPTGFK (C)
1701.8869	1701.9063	-11		65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.8630	1718.8826	-11		216	229	0	(R) NLDIERPTYTNLNR (L)
1774.9718	1774.9743	-1.4		265	280	0	(R) IHFVLTSYAPVISAEK (A)
2346.0751	2346.0137	26	1Met-ox	403	422	0	(R) AFVHWYVGEGMEEGEF SEAR (E)
2401.1790	2401.1900	-4.6		85	105	1	(R) QLFHPEQLISGKEDA ANNYAR (G)
2409.3147	2409.2091	44		244	264	0	(R) FDGALNVDLTEFQTN LVPYPR (I)

viii. Spot # 8

MOWSE Score	#/38(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
3.967e+05	**10 (26)**	**26.0**	**-2.88**	**94.4**	**49788/4.9**	**P04106**	TRYBR	TUBULIN ALPHA CHAIN

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1267.6716	1267.5096	128		312	320	0	(K) YMACCLMYR (G)
1396.7060	1396.6935	8.9		391	401	1	(R) IDHKFDLMYSK (R)
1396.7060	1396.7589	-38		85	96	0	(R) QLFHPEQLISGK (E)
1412.6729	1412.6884	-11	1Met-ox	391	401	1	(R) IDHKFDLMYSK (R)
1443.8264	1443.8535	-19		230	243	0	(R) LIGQVVSSLTASLR (F)
1525.7397	1525.7691	-19		340	352	0	(R) TIQFVDWSPTGFK (C)
1596.8015	1596.8961	-59		321	336	1	(R) GDVVPKDVNAAVATIK (T)
1681.8559	1681.8702	-8.5		339	352	1	(K) RTIQFVDWSPTGFK (C)
1701.8965	1701.9063	-5.8		65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.8814	1718.8826	-0.70		216	229	0	(R) NLDIERPTYTNLNR (L)
1774.9615	1774.9743	-7.3		265	280	0	(R) IHFVLTSYAPVISAEK (A)

ix. Spot # 9

MOWSE Score	#/35(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
1.237e+06	**9 (25)**	**27.0**	**1.06**	**33.2**	**49788/4.9**	**P04106**	TRYBR	TUBULIN ALPHA CHAIN

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1396.7162	1396.6935	16		391	401	1	(R) IDHKFDLMYSK (R)
1396.7162	1396.7589	-31		85	96	0	(R) QLFHPEQLISGK (E)
1412.6986	1412.6884	7.2	1Met-ox	391	401	1	(R) IDHKFDLMYSK (R)
1443.8188	1443.8535	-24		230	243	0	(R) LIGQVVSSLTASLR (F)
1525.7633	1525.7691	-3.8		340	352	0	(R) TIQFVDWSPTGFK (C)
1701.9020	1701.9063	-2.5		65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.9018	1718.8826	11		216	229	0	(R) NLDIERPTYTNLNR (L)
1774.9876	1774.9743	7.5		265	280	0	(R) IHFVLTSYAPVISAE K (A)
2401.2148	2401.1900	10		85	105	1	(R) QLFHPEQLISGKEDAAN NYAR (G)
2409.2549	2409.2091	19		244	264	0	(R) FDGALNVDLTEFQTNLV PYPR (I)

B. MS-FIT analysis of peptide mass lists from proteins extracted from 2D gel of KO1 biotinylated proteins isolated using avidin chromatography

i. Spot # 1

MOWSE Score	#/11(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
106	**8 (72)**	**46.0**	**-60.2**	**147**	**61813/6.1**	**33944815 M**	UNREADABLE	gi|33944815|ref|XP_340555.1|conserved/ hypothetical protein [T. brucei]

m/z Submitted	MH^+ Matched	Delta ppm	Start	End	Missed Cleavages	Database Sequence
1575.6781	1575.7420	-41	524	536	0	(R) SLQLQHIPDDESK (C)
1593.6723	1593.7769	-66	186	198	0	(K) CCPITITDFLQPR (G)
1691.7173	1691.9501	-138	463	475	1	(R) ELPLRLVVLLWEK (Y)
1707.6610	1707.7164	-32	186	198	0	(K) CCPITITDFLQPR (G)
1759.8116	1759.8776	-38	247	260	1	(K) TVSALARCLFFWSR (R)
1820.7033	1820.9481	-134	186	200	1	(K) CCPITITDFLQPRGR (A)
1851.8070	1851.8262	-10	331	350	0	(K) GNGGYGGGGGYAGDEH TVK (K)
2807.1937	2807.3649	-61	476	499	0	(K) YMAVLNSETVHDFHAYVCA VLLTR (V)
2810.1886	2810.1932	-1.6	201	225	1	(R) ASQSDWSQQGGGSLMEMH LSPDDRK (N)

ii. Spot # 6

MOWSE Score	#/35(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
1.557e+06	**16 (45)**	**26.0**	**-112**	**62.4**	**69743/6.3**	**397891 M**	TRYPANOSOMA BRUCEI	paraflagellar rod protein

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
871.4132	871.5365	-142		111	117	1	(K) KVLQDLR (Q)
930.3801	930.5009	-130		347	353	1	(R) RIEEIDR (E)
961.3923	961.5219	-135		282	289	0	(K) HIHDAIQK (A)
974.3981	974.5271	-132		410	418	0	(K) TSQDLAALR (L)
993.3574	993.4794	-123		424	430	0	(K) EHLEYFR (M)
1260.5800	1260.7316	-120		227	236	1	(R) LIDLIQDKFR (L)
1301.4831	1301.6953	-163		57	69	0	(K) AEELVAAVDVGTK (W)
1351.5373	1351.6898	-113		70	80	0	(K) WNLTEVYDLAK (L)
1354.5627	1354.7079	-107		407	418	1	(R) HDKTSQDLAALR (L)
1531.6543	1531.6712	-11		509	520	1	(K) ESEEVSGRCWHR (V)
1531.6543	1531.8154	-105	1Met-ox	485	497	1	(R) LRQGVEEELAMLK (E)
1585.6409	1585.8127	-108		419	430	1	(R) LDVHKEHLEYFR (M)
1792.7494	1792.9234	-97		359	373	1	(R) VEYSQFLEVASQHKK (L)
1807.7972	1807.9918	-108		52	69	1	(R) GAHLKAEELVAAVDVGTK (W)
1820.7601	1820.9295	-93		358	372	1	(R) RVEYSQFLEVASQHK (K)
1820.7601	1820.9944	-129		373	387	1	(K) KLLELTVYNCDLAIR (C)
1841.8282	1841.9973	-92		149	165	0	(R) SQLDATQLAQVPTQTLK (Q)
1997.8916	1998.0984	-104		148	165	1	(R) RSQLDATQLAQVPTQTLK (Q)

iii. Spot # 7

	#/32(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
1.104e+06	**10 (31)**	**32.0**	**-34.7**	**85.0**	**49788/4.9**	**P04106**	TRYBR	TUBULIN ALPHA CHAIN

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1267.6195	1267.5096	87		312	320	0	(K) YMACCLMYR (G)
1396.6579	1396.6935	-26		391	401	1	(R) IDHKFDLMYSK (R)
1396.6579	1396.7589	-72		85	96	0	(R) QLFHPEQLISGK (E)
1412.6251	1412.6884	-45	1Met-ox	391	401	1	(R) IDHKFDLMYSK (R)
1443.7674	1443.8535	-60		230	243	0	(R) LIGQVVSSLTASLR (F)
1525.6804	1525.7691	-58		340	352	0	(R) TIQFVDWSPTGFK (C)
1701.8208	1701.9063	-50		65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.8170	1718.8826	-38		216	229	0	(R) NLDIERPTYTNLNR (L)
1774.8834	1774.9743	-51		265	280	0	(R) IHFVLTSYAPVISAEK (A)

2345.9387	2346.0137	-32	1Met-ox	403	422	0	(R) AFVHWYVGEGMEEG EFSEAR (E)
2409.1220	2409.2091	-36		244	264	0	(R) FDGALNVDLTEFQTN LVPYPR (I)

iv. Spot # 9

MOWSE Score	#/30(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
5.284e+04	**7 (23)**	**22.0**	**-43.1**	**66.9**	**49788/4.9**	**71581 M**	TRYPANOSOMA BRUCEI RHODESIENSE	tubulin alpha chain

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1132.5425	1132.5672	-22		113	121	0	(K) EIVDLCLDR (I)
1396.6848	1396.6935	-6.2		391	401	1	(R) IDHKFDLMYSK (R)
1396.6848	1396.7589	-53		85	96	0	(R) QLFHPEQLISGK (E)
1443.7865	1443.8535	-46		230	243	0	(R) LIGQVVSSLTASLR (F)
1525.6985	1525.7691	-46		340	352	0	(R) TIQFVDWSPTGFK (C)
1701.8567	1701.9063	-29		65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.8395	1718.8826	-25		216	229	0	(R) NLDIERPTYTNLNR (L)
1862.6256	1862.8426	-116		312	326	1	(K) YMACCLMYRGDVVPK (D)

v. Spot # 12

MOWSE Score	#/30(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
8.581e+10	**17 (56)**	**32.0**	**-74.5**	**23.6**	**49705/4.7**	**71588 M**	TRYPANOSOMA BRUCEI RHODESIENSE	tubulin beta chain

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1041.4802	1041.5733	-89		310	318	0	(R) YLTASALFR (G)
1076.4539	1076.5489	-88		155	162	1	(K) LREQYPDR (I)
1125.5800	1125.6784	-87		253	262	0	(K) LAVNLVPFPR (L)

1146.4926	1146.5907	-86		242 251	0	(R) FPGQLNSDLR (K)
1227.5192	1227.6196	-82		381 390	0	(R) VGEQFTLMFR (R)
1243.5118	1243.6145	-83	1Met-ox	381 390	0	(R) VGEQFTLMFR (R)
1253.6697	1253.7734	-83		252 262	1	(R) KLAVNLVPFPR (L)
1274.5840	1274.6857	-80		242 252	1	(R) FPGQLNSDLRK (L)
1341.5366	1341.6439	-80		47 58	0	(R) INVYFDEATGGR (Y)
1631.7208	1631.8314	-68		63 77	0	(R) SVLIDLEPGTMDSVR (A)
1647.7168	1647.8264	-66	1Met-ox	63 77	0	(R) SVLIDLEPGTMDSVR (A)
1654.7181	1654.8238	-64		263 276	0	(R) LHFFMMGFAPLTSR (G)
1663.7367	1663.8365	-60		283 297	0	(R) GLSVPELTQQMFDAK (N)
1670.7047	1670.8187	-68	1Met-ox	263 276	0	(R) LHFFMMGFAPLTSR (G)
1686.7008	1686.8136	-67	2Met-ox	263 276	0	(R) LHFFMMGFAPLTSR (G)
1724.7500	1724.8648	-67		337 350	0	(K) NSSYFIEWIPNNIK (S)
2812.2236	2812.3596	-48		78 103	0	(R) AGPYGQIFRPDNFIFG QSGAGNNWAK (G)

vi. Spot # 16

MOWSE Score	#/40(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
5.270e+06	**10 (25)**	**28.0**	**-90.5**	**87.2**	**49788/4.9**	**71581 M**	TRYPANOSOMA BRUCEI RHODESIENSE	tubulin alpha chain

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1267.5513	1267.5096	33		312	320	0	(K) YMACCLMYR (G)
1396.5794	1396.6935	-82		391	401	1	(R) IDHKFDLMYSK (R)
1396.5794	1396.7589	-128		85	96	0	(R) QLFHPEQLISGK (E)
1412.5443	1412.6884	-102	1Met-ox	391	401	1	(R) IDHKFDLMYSK (R)
1443.6854	1443.8535	-116		230	243	0	(R) LIGQVVSSLTASLR (F)
1525.6040	1525.7691	-108		340	352	0	(R) TIQFVDWSPTGFK (C)
1681.6858	1681.8702	-110		339	352	1	(K) RTIQFVDWSPTGFK (C)
1701.7214	1701.9063	-109		65	79	0	(R) AVFLDLEPTVVDEVR (T)
1718.7032	1718.8826	-104		216	229	0	(R) NLDIERPTYTNLNR (L)
2105.8085	2105.9668	-75		41	60	0	(K) TIGVEDDAFNTFFSETGA GK (H)
2408.9835	2409.2091	-94		244	264	0	(R) FDGALNVDLTEFQTNLVP YPR (I)

vii. Spot # 20

MOWSE Score	#/46(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
2.931e+04	**8 (17)**	**11.0**	**-92.6**	**112**	**110022/5.6**	**7573281**	TRYPANOSOMA BRUCEI BRUCEI	putative coatomer beta subunit

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1163.5008	1163.4978	2.6		923	931	0	(R) YVSCNMYAR (T)
1249.4855	1249.7044	-175		729	738	1	(K) TEEFLIKLEK (T)
1267.5646	1267.7639	-157		153	163	1	(R) NAVLAVHRIFK (R)
1305.5791	1305.6989	-92		845	855	0	(R) VDIMNYVRPAK (C)
1567.5874	1567.6885	-64		191	203	0	(R) NAFEMLVECSPDR (V)
2183.8888	2184.1235	-107		572	590	1	(K) LIIRLFNHHSSGVDESMK (E)
2403.0421	2403.2165	-73		216	237	0	(K) NLESLGATLQMSIVDF HMIR (A)
2807.1393	2807.3497	-75		923	946	1	(R) YVSCNMYARTLFGDDA LVSIER (D)

viii. Spot # 32

MOWSE Score	#/36(%) Masses Matched	% Cov	Mean Err ppm	Data Tol ppm	Protein MW (Da)/pI	Accession #	Species	Protein Name
1926	**6 (16)**	**7.0**	**-138**	**106**	**138159/5.8**	**Q99279**	TRYBB	Receptor-type adenylate cyclase GRESAG 4.1 (ATP pyrophosphate-lyase) (Adenylyl cyclase)

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1208.5121	1208.6098	-81		1101	1110	0	(K) MYQLNTVPSR (N)
1300.4840	1300.7775	-226		758	768	1	(R) LLQVVISNMRK (V)
1320.4668	1320.6986	-175		1168	1177	1	(R) EKLLLPYCER (W)
1880.7610	1880.9805	-117		1101	1116	1	(K) MYQLNTVPSRNFAA LR (L)

2807.1616 2807.4554	-105	285	308	1	(K) IAQDNRTVDMYLLAP SSFQFLIK (T)
2922.1497 2922.5213	-127	101	128	0	(K) TGSGATISVIRPPSYN TTAEDIFQLGVK (Q)