THE IDENTIFICATION AND CHARACTERIZATION OF TWO UNIQUE MEMBRANE-ASSOCIATED MOLECULES OF AFRICAN TRYPANOSOMES

by

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B.Sc., University of Victoria, 1990

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

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ABSTRACT

The primary structure of a 38 kDa protein isolated from membrane preparations of African trypanosomes (*Trypanosoma brucei rhodesiense*) was determined by protein and DNA sequencing. Searching of the protein database with the trypanosome translated amino acid sequence identified glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) from various prokaryotic and eukaryotic organisms as the optimal scoring protein. Surprisingly, the eukaryotic trypanosome enzyme showed the highest degree of sequence identity with the corresponding enzyme from the prokaryote *Escherichia coli*. Using recombinant DNA techniques, the trypanosome molecule was expressed in *Escherichia coli* and found to be enzymatically active, thus confirming the identity of the molecule as an NAD^+-dependent glycerol 3-phosphate dehydrogenase. A monoclonal antibody specific for the 38 kDa protein was used to localize the enzyme to glycosomes. The enzyme has a pI of 9.0, a net charge of +9 at physiological pH and contains the peroxisome-like targeting tripeptide SKM at its C-terminus, all characteristic of glycosomal enzymes. Amino acids predicted to be involved in the NAD^+-dependent glycerol 3-phosphate dehydrogenase active site have diverged from those of the mammalian enzyme. Kinetic analyses of the trypanosome GPD and GPD from rabbit muscle showed that the Km values of the two enzymes are different. The data suggests that the trypanosome protein may be a candidate target for rational drug design. Northern and Southern blot analyses showed that the trypanosome NAD^+-dependent glycerol 3-phosphate dehydrogenase was translated from a single transcript and that only two gene copies exist, thus making this molecule an attractive target for knockout mutagenesis.

A second molecule, an abundant 11 kDa membrane protein, was also purified from African trypanosomes. This protein cross-reacted with monoclonal antibodies originally generated against the lipophosphoglycan-associated protein of *Leishmania donovani*.

Immunoblot analysis showed that the 11 kDa molecule was present in a variety of species of kinetoplastids. It was found in several species and subspecies of African trypanosomes and was present in low amounts in bloodstream forms and in larger amounts in procyclic, epimastigote and metacyclic life cycle stages. The molecule was present in procyclic trypanosome membranes at approximately 2×10^5 - 1×10^6 molecules per cell. Its wide distribution in kinetoplastids and its membrane disposition suggested a name for this class of molecules (kinetoplastid membrane protein-11) and for the molecule characterized in this thesis (trypanosome kinetoplastid membrane protein-11).

The kinetoplastid membrane protein-11 molecule was purified from *Trypanosoma brucei rhodesiense* by organic solvent extraction and octyl-Sepharose chromatography and a 14 amino acid internal peptide sequence was obtained by gas phase microsequencing. This sequence matched a translated *Leishmania donovani* kinetoplastid membrane protein-11 sequence, thus suggesting the use of the *Leishmania* sequence as a probe to select for the *Trypanosoma* gene. Screening of a trypanosome cosmid library with the *Leishmania* probe, in combination with a series of polymerase chain reaction amplifications from both genomic DNA and cDNA, allowed the determination of the entire DNA sequence and corresponding translated amino acid sequence of the trypanosome kinetoplastid membrane protein-11. The 92 amino acid sequence showed 18 percent sequence divergence from the corresponding molecule of the related kinetoplastid *Leishmania donovani donovani*, including one key amino acid at position 45 which may be of functional relevance. The secondary structure of the trypanosome molecule was predicted to form two amphipathic helices connected by a random-coil segment, and suggests that it would interact with lipid bilayers in the parasite cell membrane. Northern and Southern blot analyses using the *T.b. rhodesiense* ViTat 1.1 clone showed that the trypanosome molecule was translated from a single transcript and that there was only a single gene copy, thus making this molecule an attractive target for knockout mutagenesis.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED:

ADP, adenosine diphosphate
AMP, adenosine monophosphate
ATCC, American Type Culture Collection
ATP, adenosine triphosphate
BCIP, 5-Bromo-4-Chloro-3'-Indolyl Phosphate
BSF, bloodstream form
BLAST, Basic Local Alignment Search Tool
CATT, card agglutination trypanosomiasis test
CO_2, carbon dioxide
CoA, coenzyme A
CsCl, cesium chloride
DEAE, diethylaminoethyl
DHAP, dihydroxyacetone phosphate
DNA, deoxyribonucleic acid
ECL, enhanced chemiluminescence
EDTA, ethylene-diaminetetra-acetic acid
ELISA, enzyme linked immunosorbent assay
ESAG, expression site-associated gene
F-2,6-P_2, 2,6-bisphosphofructose
F6-P, fructose 6-phosphate
FAD, flavine adenine dinucleotide (oxidized form)
FBS, fetal bovine serum
FDP, fructose 1,6-bisphosphate
FITC, fluorescein-isothiocyanate
G-1,3-P_2, 1,3-bisphosphoglycerate
G-3-P, glycerol 3-phosphate
G6-P, glucose 6-phosphate
GAP, glyceraldehyde 3-phosphate
GARP, glutamic acid/alanine-rich protein
GIPL, glycosylinositolphospholipid
gp63, glycoprotein 63
GPD, glycerol 3-phosphate dehydrogenase
GPI, glycosylphosphatidylinositol

GRESAG, gene related to expression site-associated gene
GST, glutathione-S-transferase
HPLC, high performance liquid chromatography
ILRAD, International Laboratory for Research on Animal Diseases
IPTG, isopropyl-b-D-galactosidase
ISG, invariant surface glycoprotein
KMP-11, kinetoplastid membrane protein-11
LB, Luria-Bertani
LDL, low density lipoprotein
LPG, lipophosphoglycan
LPGAP, lipophosphoglycan-associated peptide
mAb, monoclonal antibody
MEM, minimal essential medium
MHC, major histocompatability complex
M-VAT, metacyclic-variable antigen type
NAD^+, nicotinamide adenine dinucleotide (oxidized form)
NADH, nicotinamide adenine dinucleotide (reduced form)
$NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH, nicotinamide adenine dinucleotide phosphate (reduced form)
NBT, Nitro-Blue Tetrazolium
2-PGA, 2-phosphoglycerate
3-PGA, 3-phosphoglycerate
P_i, inorganic phosphate
PAG, procyclin associated gene
PARP, procyclic acidic repetitive protein
PATT, procyclic agglutination trypanosomiasis test
PBS, phosphate-buffered saline
PCF, procyclic culture form
PCR, polymerase chain reaction
PEG, polyethylene glycol
PEP, phosphoenolpyruvate
pI, isoelectric point
PSG, phosphate-buffered saline/glucose
PSSA-2, procyclic stage surface antigen-2
3' RACE, 3' rapid amplification of cDNA ends
RNA, ribonucleic acid

SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis
SHAM, salicylhydroxamic acid
TFA, trifluoroacetic acid
THT, trypanosome hexose transporter
VAT, variable antigen type
VSG, variant surface glycoprotein
WHO, World Health Organization

ACKNOWLEDGEMENTS

Thanks to Dr. W. Robert McMaster (Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada) and Dr. William Hintz (Department of Biology, University of Victoria, Victoria, British Columbia, Canada) for providing our first introduction to the intricacies of polymerase chain reaction techniques; Dr. Ben Koop and Jackie Brinkman (Department of Biology, University of Victoria, Victoria, British Columbia, Canada) for their expert assistance in automated sequencing; Dr. Robert W. Olafson and Dr. Armando Jardim (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) for kindly providing us with the *Leishmania donovani* KMP-11 vector construct and for insightful discussions concerning the KMP-11 research project; and Tom Gore (Department of Biology, University of Victoria, Victoria, British Columbia, Canada) for his patience and help with scanning the figures. I would also like to thank the talented scientists who have contributed to work performed in this thesis, including: Dr. Isabel Roditi and Dr. Erik Vassella (Department of General Microbiology, University of Bern, Bern, Switzerland); Dr. Ute Frevert and Gabrielle Pradel (Department of Medical and Molecular Parasitology, New York University Medical Center, New York, New York, USA); Dr. Tom Mommsen, Glenn Cooper, Rob Beecroft, Cory Tuckey, Gerry Baron and Mike Bridge (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada).

Thanks also to Albert Labossiere and Scott Scholz for their technical expertise, reliability and friendship and to all the departmental faculty and staff whom I have had the pleasure to work with over the years. Special thanks go to my supervisory committee, who have provided me with expert guidance throughout this period of study and who have invariably been positive, helpful and supportive in their attitudes.

I am indebted to The University of Victoria, The Arthritis Society of British Columbia (Victoria Branch) and The Natural Sciences and Engineering Research Council of Canada for financial support in the form of fellowships, scholarships and research grants.

To the wonderful people, past and present, with whom I have had the opportunity to work with in Terry's lab, you have truly made it a pleasure to come to work every day. Thanks also to my many friends whom I hold close to my heart. I am most grateful to my

superb family, my parents Keith and Eva-Lind Cameron, my sisters Anne and Susan, and my brothers Paul, John, Gordon, Ian and Kim; your unconditional love and support have made me believe I could attain any goal in life. Particular recognition goes to my mom, who has instilled in me the importance of learning through her subtle, but constant, quest for knowledge.

And to Terry, you deserve the most special thanks of all. Through your wisdom, support, enthusiasm and friendship you have been, and always will be, a true mentor to me.

Caroline

GENERAL INTRODUCTION

The first documented observation of a trypanosome was made in 1841 in Berne, Switzerland by Professor Gabriel Valentin, who noted "a dark, motile, bullet-shaped object lying between the blood cells" upon microscopic examination of the blood of a trout [Valentin, 1841]. The term "trypanosome" was coined in 1843 by David Gruby who likened the organism's motility to that of an auger or corkscrew (trupanon; Greek for borer). Since this initial discovery these protozoan parasites have been the focus of intense research, due in part to their exquisite uniqueness in the realm of the eukaryotic world and their suitability for use as model organisms for biochemical, molecular biological and immunological research. Particular emphasis has been given to the medically and socio-economically significant African trypanosomes, which cause sleeping sickness in humans and Nagana (from the Zulu, meaning poorly) in cattle. Although African sleeping sickness was first described in the fourteenth century by the Arab writer al Qualquashaudi [Hoeppli, 1959], the African trypanosomes themselves were not identified until the latter part of the nineteenth century. *Trypanosoma evansi*, which infects camels and horses, was the first to be discovered in 1880 by Griffith Evans. Subsequent work by David Bruce in 1894 identified *T. brucei brucei* as the organism responsible for causing "fly disease" in cattle, a monumental contribution that first revealed the intimate connection between the disease, the tsetse fly and the trypanosome. *T. brucei gambiense* was discovered in 1902 by J. E. Dutton and was theorized by Maxwell Adams in 1903 to be the causative agent of African sleeping sickness in humans, a prediction later validated by a medical commission consisting of David Bruce, Aldo Castellani and D. N. Nabarro. *T. brucei rhodesiense*, the second causative agent of African sleeping sickness in humans, was discovered in 1910 by J. W. W. Stephens [Historical information from Hoare, 1972].

No other disease has had more impact on the development of an entire continent than that of trypanosomiasis on Africa. In fact, the World Health Organization (WHO) recognizes the African trypanosome to be one of the most important parasites affecting the people of the tropical Third World [WHO, 1991]. Human trypanosomiasis has been responsible for hundreds of thousands of deaths in great epidemics; in the late 1890s and early 1900s trypanosomiasis decimated over two thirds of the population living around the north shore of Lake Victoria [McKelvey, 1973]. Although such large epidemics no longer occur due to the implementation of expensive and elaborate tsetse fly control measures, African sleeping sickness is still medically relevant with more than 50 million

people at risk from the disease and 20,000 new cases reported annually. In addition, disruption of medical services and population movements caused by social instability have prompted the occurrence of recent small-scale disease epidemics [Cattand, 1988]. Perhaps the greatest impact of African trypanosomes is from the uncontrolled state of animal trypanosomiasis. More than 200 million cattle are at risk of Nagana, a bovine disease characterized by slow growth, weight loss, poor milk yield, infertility and death. Moreover, bovine trypanosomiasis renders 11 million square kilometers south of the Sahara unsuitable for cattle grazing [Murray et al., 1990]. As a result, Nagana contributes to human malnutrition and negatively influences the African economic environment. Certain wildlife species indigenous to Africa have evolved resistance to the same trypanosomes responsible for causing African sleeping sickness and Nagana, and thus represent a perpetual reservoir for the disease [Vickerman et al., 1993].

1. Biology and Life Cycle of the African Trypanosomes

1.1 Classification

The African trypanosomes are unicellular eukaryotic organisms that belong to the genus *Trypanosoma*; family Trypanosomatidae; suborder Trypanosomatina; order Kinetoplastida; class Zoomastigophorea; superclass Mastigophora; subphylum Sarcomastigophora; phylum Protozoa. Members of the genus *Trypanosoma* are digenetic parasites living alternately in the bloodstream and tissues of vertebrates and the gut of leeches or arthropods. The family Trypanosomatidae consists of a great variety of genera that infect mammals, birds, fish, amphibia, insects and plants. The parasites of medical importance all belong to the two genera *Trypanosoma* and *Leishmania* [Vickerman, 1978]. The genus *Trypanosoma* is split into two divisions (*Stercoraria* and *Salivaria*) based primarily upon the organism's developmental course within the insect vector. The *Stercoraria* division includes species whose terminal developmental stage occurs in the posterior gut of the insect vector and are transmitted through the fecal route, such as the etiologic agent of Chagas' disease, *Trypanosoma cruzi*. The *Salivaria* division consists of species that complete development in the anterior part of the vector's digestive tract and are transmitted via vector saliva [Hoare, 1972]. The African trypanosomes correspond to this division and thus are referred to as salivarian trypanosomes. The three major pathogenic subgenera of this division are *Duttonella* [type species *Trypanosoma*

(*Duttonella*) *vivax*], *Nannomonas* [type species *Trypanosoma* (*Nannomonas*) *congolense*] and *Trypanozoon* [type species *Trypanosoma* (*Trypanozoon*) *brucei*].

1.2 Biology

African trypanosomes possess a number of biochemical peculiarities that distinguish them from all other eukaryotic organisms. These unique characteristics contribute not only to the success of their parasitic lifestyle but also to their popularity as targets for research. In historic order these features include:

1. The ability to alter the antigenic composition of the variant surface glycoprotein (VSG) surface coat and thereby evade the host's immune response. This process is called antigenic variation and the African trypanosomes represent the prototype for this phenomenon.

2. The possession of a kinetoplast, which constitutes part of the mitochondrion and contains a highly unusual form of mitochondrial DNA, known as kinetoplast DNA, that forms an enormous network of catenated circles. Within the kinetoplast additional unique characteristics are observed, including the widely studied RNA editing which results in the addition and removal of uridine residues from certain mRNA transcripts [Eisen, 1988; Benne, 1989; Simpson and Shaw, 1989; Stuart, 1989; Benne, 1990; Simpson, 1990; Stuart et al., 1990; Weiner and Maizels, 1990; Feagin, 1991; Stuart, 1991a; Stuart, 1991b]. As the name suggests, kinetoplast DNA is found in all representatives of the order Kinetoplastida.

3. The compartmentalization of the glycolytic pathway from glucose to 3-phosphoglycerate within a microbody-like organelle termed the glycosome [Opperdoes and Borst, 1977]. This organelle is common to the order Kinetoplastida and will be discussed in depth in Chapter 1.

4. Discontinuous transcription of protein-coding genes in which RNA segments from two transcription units are joined [Borst, 1986]. This also represents a shared feature of all members of the order Kinetoplastida.

Due to the central role antigenic variation plays in host immune evasion, and consequently parasite survival and disease establishment, this unique trait of African

trypanosomes will be considered in more detail. This process represents one of the most studied aspects of African trypanosomes, and numerous comprehensive reports have been made on this subject [Borst and Cross, 1982; Borst, 1983; Borst et al., 1983; Bernards, 1985; Boothroyd, 1985; Donelson and Rice-Ficht, 1985a; Donelson and Turner, 1985b; Turner et al., 1985; Borst, 1986; Pays and Steinert, 1988; Cross, 1990]. The surface coat of each trypanosome is composed of a single VSG type, the structure of which determines the parasite's variable antigen type (VAT) [Cross, 1990]. The relapsing parasitemia characteristic of African sleeping sickness is caused in part by host humoral responses to antigenically distinct VSGs; antibodies produced against the VSG react only with parasites of the same VAT. Therefore, while the host mounts a strong immune response against the major VATs in the trypanosome population and thus eliminates these parasites from the bloodstream, a small number of parasites in the population bear an antigenically distinct VSG and as a result escape immune destruction. These surviving parasites proliferate and go on to establish the next parasitemic wave, which in turn induces a VAT-specific immune response [Vickerman, 1978]. This process continues, resulting in the characteristic fluctuating parasitemia.

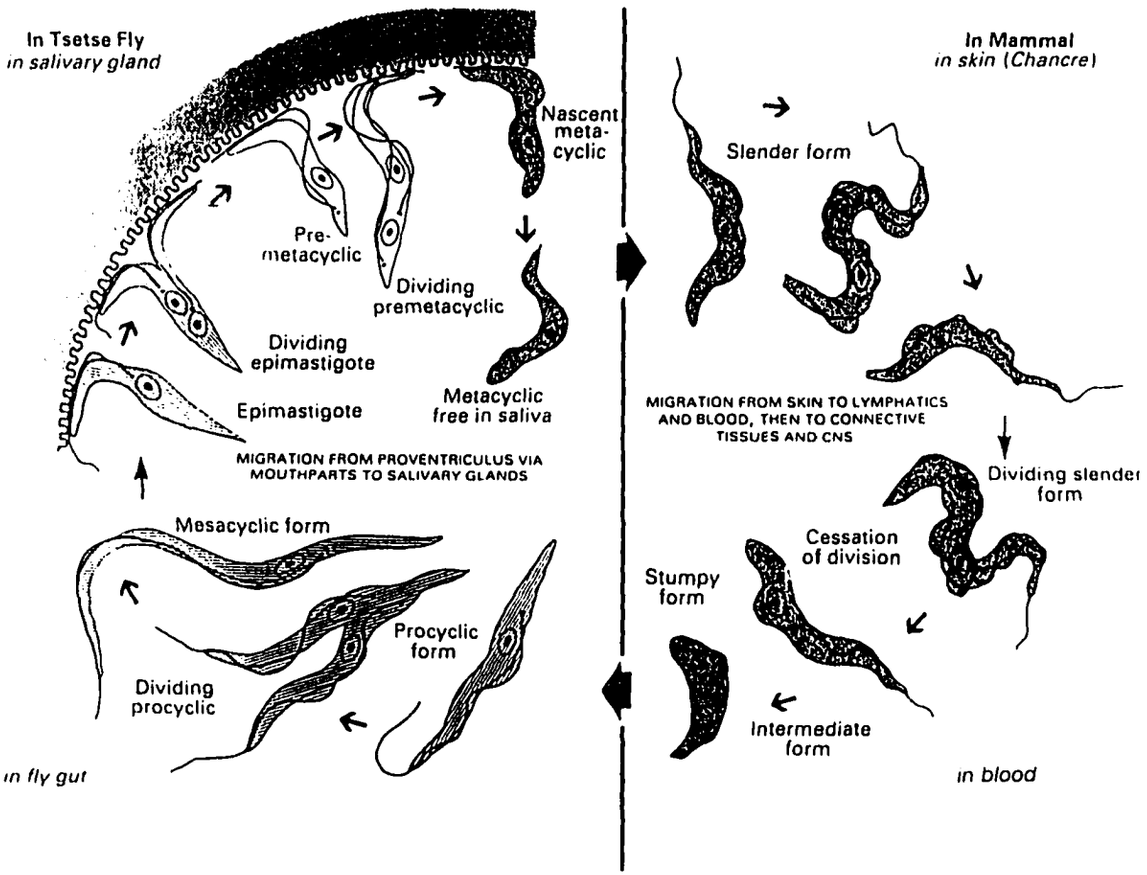
Infections initiated with a single trypanosome produce antigenically distinct variants [Ritz, 1916], demonstrating that variation is a property of an individual trypanosome. Each VSG is encoded by a separate gene and there are approximately 10^3 VSG genes (and pseudogenes) per trypanosome nucleus [Van der Ploeg et al., 1982]. Although there is a strong tendency for some VSGs to be expressed early in infection and for others to appear late, the sequence of VSG expression is by no means ordered [Van Meirvenne et al., 1975; Capbern et al., 1977; Miller and Turner, 1981]. Taken together, these findings demonstrate the extraordinary complexity of VSG expression during a trypanosome infection. This exemplifies the purported function of the VSG surface coat; that is, to present an immunogenic, unified front to the host's immune system and thus detract from any underlying, shielded, functionally essential molecules present on the cell surface. In addition, antigenic variation and the host immune response serves to control the extent of parasitemia and, hence, promote the development of long-lasting chronic infections to ensure parasite survival through increased probability of transmission to the tsetse vector and ultimately to a new mammalian host.

1.3 Life Cycle

The majority of this thesis deals with research performed on the African trypanosome *Trypanosoma brucei* spp; consequently, only the life cycle of these protozoans will be discussed. *T. brucei* spp. represent the most widely studied African trypanosomes and are divided into the three aforementioned subspecies of veterinary and medical significance, *T. brucei brucei*, *T. brucei gambiense* and *T. brucei rhodesiense*. These subspecies are biochemically and morphologically identical but differ in host range and virulence [Hoare, 1972]. *T. brucei brucei* is noninfectious to humans, at least in part because it is lysed by a species of high density lipoprotein in human serum [Rifkin, 1978]. *T. brucei gambiense* is distributed mostly in West and Central Africa and causes the more chronic illness (death within 2-3 years without treatment), while *T. brucei rhodesiense* is found mostly in Southern and East Africa and causes the more acute disease (death in less than a year without treatment) [Hoare, 1972].

The life cycle of *T. brucei* spp. is one of the most complex cycles described for these hemoflagellates (Figure 1). During its developmental cycle the trypanosome alternates between proliferative phases in which it undergoes binary fission, and non-proliferative phases in which it is incapable of division. The latter are associated with major transitions in environment, the former with establishing the parasite in the new environment [Vickerman, 1985]. Infection is initiated in the mammalian host by the bite of a trypanosome-infected bloodsucking tsetse fly (*Glossina* spp.), with the simultaneous injection of non-dividing metacyclic trypanosomes into the dermal connective tissue from the vector's saliva. This life cycle stage is preadapted for life in the mammalian host in that it possesses the VSG surface coat [Tetley et al., 1987]. The extruded metacyclic population is heterogeneous with respect to VAT but the repertoire of VATs found is limited, with no more than 27 observed for *T.b. rhodesiense* [Turner et al., 1988]. Within the dermis the trypanosomes differentiate to the actively dividing long-slender bloodstream form (BSF) [Ormerod, 1970] which has the potential to express the full repertoire of VATs [Van der Ploeg et al., 1982]. The trypanosomes subsequently escape from the bite site and enter the draining lymphatics and then the bloodstream. *T. brucei* spp. parasites are pleomorphic in the blood, multiplying with a 4-6 hour generation time [Herbert and Parratt, 1979; MacAskill and Holmes, 1983] as long-slender BSF trypanosomes in the ascending parasitemia and transforming through an intermediate bloodstream stage to the non-dividing short-stumpy BSF as the parasitemic wave passes through the crest and goes into remission [Vickerman et al., 1993]. The short-stumpy

Figure 1 Diagram of the life cycle of *Trypanosoma brucei* spp. The phases of multiplication and attachment and changes in the immunodominant surface proteins are indicated. VSG coat: stippled. Procyclin coat: cross-hatched. [From Vickerman et al., 1993].


In Tsetse Fly
in salivary gland
Nascent meta-cyclic
Pre-metacyclic
Dividing premetacyclic
Dividing epimastigote
Epimastigote
Metacyclic free in saliva
MIGRATION FROM PROVENTRICULUS VIA MOUTHPARTS TO SALIVARY GLANDS
Mesacyclic form
Procyclic form
Dividing procyclic
in fly gut
In Mammal
in skin (Chancre)
Slender form
MIGRATION FROM SKIN TO LYMPHATICS AND BLOOD, THEN TO CONNECTIVE TISSUES AND CNS
Dividing slender form
Cessation of division
Stumpy form
Intermediate form
in blood

BSF does not undergo antigenic variation [Vickerman, 1985] and is believed to be preadapted to life in the insect vector [Vickerman et al., 1993]. The parasites may secondarily escape from the bloodstream into the soft connective tissues and multiply in the tissue fluid. Invasion of the brain and cerebrospinal fluid occurs in chronic infections.

The cycle of development in the fly is initiated when a tsetse fly takes a blood meal from an infected mammal. The infected blood is ingested by the fly into the crop and then into the lumen of the midgut where the short-stumpy trypanosomes transform into the procyclic stage; slender forms die or transform into the short-stumpy form in the anterior midgut. Transformation to procyclics takes place in the posterior part of the midgut in the endoperitrophic space (located inside the chitinous membrane that separates the blood meal from the midgut epithelium) and is accompanied by morphological changes as the trypanosome adapts to life in the tsetse vector. These include an increase in body length and elaboration of the simple mitochondrion to satisfy the parasite's respiratory requirements within the vector (discussed in detail in Chapter 1). The changes occur over a 48-72 hour period in the tsetse gut, and during this time active division of the parasites is observed [Vickerman, 1985]. Concurrent with transformation is the progressive loss of VSG from the parasite surface and the simultaneous replacement with the stage-specific surface glycoproteins, the procyclins. Thus at no time during differentiation is the parasite uncoated [Roditi and Pearson, 1990]. The procyclic trypanosomes, which are devoid of a VSG coat and uniformly possess a procyclin coat, subsequently penetrate the peritrophic membrane and invade the ectoperitrophic space. As the parasites move forward to the proventriculus, they elongate further, cease to divide and differentiate to proventricular 'mesocyclic' trypanosomes. The mesocyclics then reinvade the endotrophic space and undertake an elaborate journey via the esophagus, mouthparts and salivary ducts to establish infection in the salivary glands [Vickerman, 1985].

The salivary gland trypanosome population proceeds through four sequential developmental stages. The multiplicative epimastigote stage bears the procyclin surface coat and is attached to gland cell microvilli by branched outgrowths of the flagellum [Vickerman, 1985]. Dividing premetacyclic trypomastigotes lack both procyclin and VSG surface coats [Vickerman et al., 1993] and are still anchored to gland cells despite exhibiting microvilli-attachment outgrowths that are much reduced in size [Vickerman, 1985]. Nascent metacyclics have re-acquired the VSG surface coat and have ceased division but are still attached, whereas mature, non-dividing metacyclic trypanosomes lie free in the saliva of the tsetse fly [Vickerman, 1985; Vickerman et al., 1988; Vickerman et

al., 1993]. Both nascent and mature metacyclics possess an unbranched mitochondrion [Vickerman, 1985], a respiratory adaptation for life in the mammalian host that will be discussed in detail in Chapter 1.

The entire developmental cycle within the tsetse fly takes 3-5 weeks. The success of completion of the life cycle within the tsetse fly is not absolute; the complexity of *T. brucei* spp. development causes many infections to abort and only produces metacyclics in 2-5% of tsetse flies [Vickerman et al., 1988]. In fact, the presence of a lectin defense system in the tsetse gut which greatly reduces the number of active trypanosomes capable of continuing the infection has been demonstrated [Maudlin and Welburn, 1987]. However, trypanosome evolutionary ingenuity has compensated for this defense by utilizing a symbiont-induced characteristic observed in *Glossina morsitans*. Rickettsia-like symbionts reside in the tsetse midgut and produce a chitinase which promotes D^+-glucosamine release. This sugar is thought to interfere with the lectin defense system and the enzyme itself is believed to enhance penetration of the peritrophic membrane by procyclic trypanosomes, thus increasing the susceptibility of *Glossina morsitans* to *T. brucei* spp. infection [Maudlin and Ellis, 1985].

1.4 African Trypanosomes in the Laboratory

Cultivation techniques for salivarian trypanosomes have significantly advanced in recent years such that all representative life cycle stages can be obtained. Laboratory rodents can be infected with syringe-passaged BSF trypanosome populations. It should be stressed, however, that such mechanically transmitted-infections of rodents may not truly mimic cyclically transmitted-infections of the natural host. Specifically, changes that can occur in syringe-passaged trypanosomes include an increase in virulence and VAT stabilization [Vickerman et al., 1993]. Also, the pleomorphic nature of *T. brucei* spp. stocks can be lost during repeated mechanical passage through laboratory rodents, creating monomorphic trypanosome lines that are unable to differentiate to the short-stumpy BSF and thus are not fly transmissible [Gray et al., 1987; Vickerman et al., 1993]. Therefore, serious consideration must be given to the trypanosome stock used for experimentation to ensure maintenance of vector-infectability.

Pleomorphic bloodstream trypanosomes can be induced to undergo differentiation *in vitro* to the procyclic life cycle stage by a shift in temperature (37°C to 26°C) and incubation in a cell-free medium supplemented with 3 mM *cis*-aconitate. Commitment of

cells to transformation takes place after 24 hours [Vickerman et al., 1988]. The procyclic life cycle stage can be easily propagated *in vitro* in a variety of cell-free media [Gray et al., 1987; Vickerman et al., 1993]. Procyclic trypanosomes cultured *in vitro* (so called procyclic culture forms, PCF) and those found in the tsetse fly midgut have been demonstrated to be morphometrically, ultrastructurally and biochemically identical [Richardson et al., 1986]. Subsequent life cycle stages of *T. brucei* spp. (representing other stages in the insect vector) are more difficult to culture due to the requirement for a substratum of living cells to allow attachment of mesocyclic trypanosomes and transformation to infective metacyclics [Gray et al., 1987]. Such a culture system *in vitro* has yet to be devised. However, cultivation *in vitro* of the entire developmental cycle of the cattle-infective *Trypanosoma congolense* is possible as this trypanosome species undergoes metacyclic differentiation via attachment to the chitinous wall of the tsetse fly proboscis. This surface can be effectively mimicked by placement of a plastic base or floating plastic coverslips within a culture flask [Vickerman et al., 1988]. In this system metacyclic trypanosomes are produced as free-swimming trypomastigotes in culture media. Addition of mammalian feeder layer cells to the culture system promotes differentiation into bloodstream forms, thus completing the developmental cycle *in vitro* [Gray et al., 1987]. Bloodstream forms can be propagated *in vitro* in the absence of feeder cells using a modified Iscove's medium containing a low concentration of serum proteins [Hirumi and Hirumi, 1989]. Clonal growth of bloodstream- and insect-form trypanosomes within agarose is also possible [Carruthers and Cross, 1992].

2. Disease Manifestations and Host Immune Responses to Infection

Intimately associated with progression of trypanosomiasis is the concomitant immune response of the mammalian host to parasitic infection, and thus disease manifestations and host immune responses to infection must be jointly considered. The immune system plays a central role in both control of parasitemia and disease pathogenesis, and the precise contribution of immune effector mechanisms to each is often complex, intertwined and incompletely understood. Additionally, the majority of studies on the cellular immunology of trypanosomiasis have been performed using the murine model and, as with all animal models of disease, care must be taken in extrapolating observations from such studies to the natural mammalian hosts. Despite these shortcomings, knowledge of disease progression and host immune effector

mechanisms is thought to be essential for the efficient development of disease control measures.

2.1 Disease Manifestations

African trypanosomiasis proceeds through three different stages of disease development. The first stage is characterized by the formation of a chancre at the tsetse fly bite site. In the second stage the trypanosomes become systemically distributed throughout the bloodstream and tissues of the mammalian host. This stage of infection is characterized by non-specific signs of infection, including intermittent fever, joint pains and general malaise, all of which result from the fluctuating parasitemia and subsequent immunological and physiological responses to high parasite load and disruption of immunoregulation (discussed below). In the third stage of the disease the parasites invade the central nervous system and cause meningoencephalitis. In humans this tertiary disease stage is characterized by irritability, psychosis and finally by loss of consciousness and is terminal unless drug treatment ensues. In cattle the main causes of death are anaemia and cachexia (wasting) which cause severe weakness and an inability to forage for food. In both humans and cattle, disease manifestations are accompanied by immunodepression (discussed below) and thus death from secondary infections may also result [Pearson, 1990].

2.2 Host Immune Responses During Infection

(i) The Chancre

Within 7 to 11 days of the bite of an infected tsetse fly a nodular lesion, termed a chancre, develops at the bite site and then gradually subsides within one month of infection [Vickerman et al., 1993]. The chancre is clearly a response to the trypanosome as bites from uninfected tsetse flies do not elicit such a lesion [Emery and Moloo, 1980; Akol and Murray, 1982]. The chancre results from a combination of an acute inflammatory response and an immune reaction to the locally proliferating trypanosomes [Shapiro and Pearson, 1986]. The chancre is characterized histologically by an initial infiltrate of polymorphonuclear leucocytes and small lymphocytes, followed by the appearance of lymphoblasts at the peak of the reaction and macrophages and plasma cells as the lesion begins to subside [Shapiro and Pearson, 1986; Pearson, 1990; Vickerman et al., 1993]. Studies of cattle infections show chancre size appears to correspond to the

extent of parasite proliferation at the bite site, the parasite species and the extent of cellular infiltration [Pearson, 1990; Vickerman et al., 1993]. The major cattle pathogens *T. brucei brucei* and *T. congolense*, which rapidly proliferate at the tsetse fly bite site, induce chancres up to 10 cm in diameter. In *T. vivax* infections of cattle, however, fewer metacyclics are injected at the bite site and these parasites tend to quickly escape to the draining lymph nodes; as a result, this trypanosome species elicits a smaller, quickly regressing chancre or no reaction at all [Vickerman et al., 1993].

The exact role of the immune response within the chancre is not fully understood. The dividing trypanosomes found throughout the chancre express the limited metacyclic VAT repertoire (designated M-VAT). It is believed that antibodies against the M-VAT are generated within the lesion, since cattle challenged with the homologous serodeme (i.e. parasites expressing the same M-VAT repertoire) fail to develop a chancre [Vickerman et al., 1993]. Therefore, immunity induced at the chancre phase may serve to influence the distinct parasite antigenic types that invade the bloodstream and elicit the early systemic response.

(ii) The Early Systemic Response

In the second stage of the disease the trypanosomes proliferate extracellularly in the bloodstream and become widely distributed throughout the host. Concurrent with the characteristic undulating waves of parasitemia is development of an immunoproliferative reaction in several lymphoid organs [Shapiro and Pearson, 1986; Pearson, 1990; Vickerman et al., 1993]. Most striking is the B-lymphocyte proliferation observed in the lymph nodes, bone marrow and spleen [Mayor-Withey et al., 1978; Morrison et al., 1981a; Morrison et al., 1981b; Morrison et al., 1982; Greenwood and Whittle, 1980]. This proliferative response is polyclonal in nature and results in a profound increase in circulating IgM [Luckins and Mehlitz, 1976; Whittle et al., 1977] directed against antigens of parasite, host and non-parasite, non-host origin [Hudson et al., 1976; Greenwood and Whittle, 1980]. Although the reason for this polyclonal response is not known it is hypothesized to arise from production or induction of a B-lymphocyte mitogen by the BSF trypanosomes [Urquhart et al., 1973; Greenwood, 1974]. In support of this hypothesis, activated macrophages from mice injected with lethally irradiated trypanosomes [Grosskinsky and Askonas, 1981] or with trypanosome membranes [Sacks et al., 1982] have been shown to induce non-specific B-lymphocyte proliferation.

Antibodies effective in elimination of trypanosomes from the bloodstream are primarily IgM and are directed to VSG epitopes exposed on the trypanosomes surface [Sendashonga and Black, 1982]. This anti-VSG antibody response has been demonstrated in nude mice and therefore is predicted to be mainly T lymphocyte independent. An anti-VSG IgG response is apparent but does not arise until after the parasites have been eliminated from the bloodstream [Clayton et al., 1979]. Trypanosomes are cleared from the bloodstream by a combination of complement-mediated lysis [Murray and Urquhart, 1977] and antibody-dependent phagocytosis [Greenblatt et al., 1983; Ngaira et al., 1983]. *In vitro* experiments utilizing guinea pig serum as a source of complement have shown long-slender BSF are lysed within 30 minutes, while the short stumpy BSF takes up to 2 hours [Barry and Vickerman, 1977]. These results parallel observations *in vivo* which demonstrate that short-stumpy forms are more resistant to the host's immune response and consequently persist into the next VSG-specific parasitemic wave, thus increasing their chances of being ingested by the vector [Balber, 1972]. This increased resistance has been proposed by Vickerman et al. [1988] to result from changes in surface membrane composition of the stumpy forms, specifically by stiffening of the membrane through an increased cholesterol content.

Early control of parasitemia within this initial systemic phase appears to be critical for establishment of relative resistance to trypanosome infection. The importance of a strong antibody response to parasitemia control has been well established through studies performed on breeds of cattle that exhibit varying degrees of susceptibility to infection. N'dama cattle are more trypanotolerant than Zebu cattle; N'dama cattle have a greater ability to control the initial wave of parasitemia [Trail et al., 1989] and exhibit a higher circulating B cell count during infection [Ellis et al., 1987] than Zebu cattle. However, the factors contributing to parasitemia control are far from clear. Experimental infections of mice have provided some insight into the role that antibody plays. Different strains of mice exhibit varying degrees of susceptibility to disease, with some strains dying within a few days of infection (i.e. C3H/He) and others surviving for several months (i.e. C57BL/6) [Pearson, 1990; Vickerman et al., 1993]. Studies performed on major histocompatability complex (MHC) congenic mice (strains which differ only in their H-2 haplotypes) have shown that resistance to *T.b. rhodesiense* and *T.b. gambiense* is multigenic and complex [Morrison and Murray, 1979; De Gee et al., 1988] and is not MHC-linked [Morrison and Murray, 1979; Levine and Mansfield, 1981]. Crosses between resistant and susceptible strains of mice produced offspring that elicited anti-VSG antibody responses and controlled the first peak of parasitemia but exhibited a

survival time similar to that of the susceptible parental strain, thus demonstrating that production of anti-trypanosome antibodies and resistance are two separate phenomena [Levine and Mansfield, 1984]. Furthermore, genetic analyses have clearly shown that different genes are responsible for resistance and for controlling parasitemia via anti-VSG antibody responses [De Gee et al., 1988; Seed and Sechelski, 1989]. Collectively these findings demonstrate that, although antibody responses play an important role in host resistance to trypanosomes, additional host factors also contribute.

The differentiation of long-slender bloodstream trypanosomes to the short-stumpy form also clearly contributes to limiting parasitemia. Experimental infections of mice with monomorphic trypanosome lines inevitably results in death [Newson et al., 1990]. The morphological transition to short-stumpy form parasites can occur in immunosuppressed animals [Balber, 1972] and therefore is not thought to be induced by specific immune responses, but instead has been suggested to result from non-immunological host factors [Newson et al., 1990]. Differentiation to the short-stumpy form has been proposed by Seed and Sechelski [1989] to be due to a parasite-induced exogenous growth inhibitor, whereas Black et al. [1985] attributed differentiation to depletion of a host-derived growth factor. However, neither situation may exist *in vivo* as superimposition of a second infection of a different VAT onto a primary infection at a time when stumpy forms predominate in the blood does not prevent multiplication of the challenge parasites as slender forms [McLintock, 1990].

The relationship between the trypanosome life cycle, the host's immune response and control of parasitemia warrants further study as it will presumably provide insight into mechanisms for control of trypanosomiasis.

(iii) The Late Systemic Response

The late systemic response is characterized by immunodepression resulting from chronic parasitemia [Shapiro and Pearson, 1986; Pearson, 1990]. This tertiary phase of the immune response to trypanosome infection has been demonstrated in sleeping sickness patients and cattle [Askonas, 1985], although it has been best studied in the murine model. In trypanosome-infected mice the state of immunodepression affects both humoral and cell-mediated responses [Corsini et al., 1977; Jayawardena and Waksman, 1977; Pearson et al., 1978; Wellhausen and Mansfield, 1980; Kar et al., 1981]. The immunodepression is caused primarily by a parasite-induced effect on macrophages. A

relative depletion of macrophages bearing Ia antigens (MHC class II molecules) is observed in lymph nodes and spleens of chronically infected mice [Bagasra et al., 1981]. This situation is thought to arise either through trypanosome production of a factor inhibitory for granulocyte and macrophage differentiation [Kaaya et al., 1979; Kaaya et al., 1980] or through decreased myeloid cell production as a result of competition with cells in the erythrocyte pathway for precursors in the bone marrow [Valli et al., 1979]. This latter state would result from the severe anemia that is characteristic of trypanosomiasis (discussed below under immunopathology). The parasite-induced effect on macrophages appears to be two-fold, since it has also been demonstrated that the limited macrophages present in the lymph node and spleen actively suppress immune responses or fail to process or present antigen properly [Askonas, 1985; Paulnock et al., 1988].

The overall effect of the macrophage dysfunction induced by trypanosome infection is depression of T lymphocyte-dependent immune responses and maintenance of T-independent B lymphocyte responses. This in turn severely retards the ability of the host to ward off secondary infections from other organisms, while trypanosome-specific anti-VSG antibody responses remain unaffected. Late in infection even the T-independent antibody responses are inhibited and trypanosomes are not effectively cleared from the bloodstream. This may represent an evolutionary adaptation of the trypanosome to limit host autoimmune responses while prolonging the time interval for transmission to the tsetse vector [Pearson et al., 1978; Mitchell and Pearson, 1983].

2.3 Immunopathology

Various pathological effects result from the profound immune responses incurred during infection with African trypanosomes. These include cytokine imbalance, immune complex formation and anemia. Macrophage dysfunction induces overproduction of tumor necrosis factor, which is responsible for the characteristic wasting (cachexia) associated with trypanosomiasis [Buetler and Cerami, 1988]. Immune complex formation results from the vast increase in circulating polyclonal immunoglobulins observed during infection and the concurrent repeated destruction of trypanosome populations [Vickerman et al., 1993]. Such complexes promote the generation of pharmacologically active substances, either by activation of the kallikrein-kinin enzyme cascade or by release from platelets [Boreham, 1979], and the ensuing inflammatory reactions lead to disruption of tissue architecture [Morrison et al., 1983] and marked

circulatory disturbances [Boreham, 1979]. Immune complexes also contribute to disease pathology by adhering to the surface of various cell types and activating complement, with the resultant destruction of these cells by lysis or opsonization [Vickerman et al., 1993].

Of paramount importance is the severe anemia that develops early in infection; in Nagana anemia is the disease manifestation that inflicts the most damage on the host [Vickerman et al., 1993]. Anemia is postulated to arise primarily by two different mechanisms. In the first situation antigen-antibody complexes passively adhere to the erythrocyte membrane, rendering these red blood cells more susceptible to phagocytosis and complement-mediated lysis [Kobayashi et al., 1976; Amole et al., 1982; Vickerman et al., 1993]. A second mechanism for red cell destruction involves the transfer of the trypanosome VSG antigen from the phospholipid bilayer of the parasite's surface to the membrane of the red blood cell, thus making these cells more susceptible to lysis by complement in the presence of VSG-specific antibodies [Rifkin and Landsberger, 1990].

The polyclonal B lymphocyte proliferation induced by trypanosome infection increases the risk for development of host autoimmune reactions. Numerous types of antibodies directed against self components have been reported to be associated with infection, including antibodies against a wide variety of tissues and cell components (erythrocytes, liver, heart, brain, kidney, thymus, DNA and RNA) [Mansfield and Kreier, 1972; Kobayakawa et al., 1979; Anthoons et al., 1986]. However, the significance of the presence of such autoantibodies to disease pathogenesis is not understood [reviewed in Shapiro and Pearson, 1986; Pearson, 1990].

3. Disease Diagnosis, Treatment and Prophylaxis

3.1 Disease Diagnosis

Early diagnosis of trypanosome infections is essential for efficient drug treatment and subsequent disease resolution. Apart from diagnosis based upon the presence of the parasites in the blood and characteristic disease symptoms, the primary test used for human sleeping sickness is the card agglutination trypanosomiasis test (CATT). Additional tests not yet adapted to field use are the procyclic agglutination trypanosomiasis test (PATT) [Pearson et al., 1986] and a double antibody sandwich

enzyme-linked immunosorbent assay [Liu and Pearson, 1987]. Most methods of disease detection have serious flaws. Parasitological diagnosis is based on detection of parasites by light microscopy in blood films or lymph node punctures, and the effectiveness of this technique can be complicated by low parasite numbers between successive parasitemic waves [Doyle, 1977; Shapiro and Pearson, 1986]. Symptom-based diagnoses are often missed, as a correlation between an insect bite and subsequent disease symptoms is usually not made. Also, in humans, symptoms may not be apparent until the later stages of disease [Apted, 1970]. The CATT [Magnus et al., 1978] utilizes a fixed, stained BSF of *T.b. gambiense* that expresses a ubiquitous VSG, and therefore this test does not detect patients infected with trypanosomes that have not expressed that specific VSG-type and is invalid for patients infected with *T.b. rhodesiense* [Liu and Pearson, 1987]. The PATT uses living *T.b. rhodesiense* PCF in an agglutination format and detects anti-procyclic antibodies in patient's sera [Pearson et al., 1986]. This test detects both *T.b. rhodesiense* and *T.b. gambiense* infections. The double antibody sandwich enzyme-linked immunosorbent assay detects circulating trypanosomal antigens in patient's sera [Liu and Pearson, 1987]. This test has been shown to be predictive of relapse infections and is thus thought to offer advantages over the antibody detection tests. The latter two diagnostic tests have a more widespread applicability than the CATT, but they have yet to be adapted to simple formats for use in the field [Pearson, 1990]. The development of new diagnostic assays, or enhancement of pre-existing ones, is required for efficient diagnosis of trypanosomiasis.

3.2 Drug Treatment

The basic requirement for chemotherapeutic treatment of African trypanosomiasis was eloquently summarized by Paul Ehrlich in 1907 when he stated "We are looking for chemical agents, which on the one hand will be taken up by and destroy certain parasites, and which on the other hand, in the amounts necessary for this destruction, will be tolerated by the organism without too much damage" [Williamson, 1970]. Despite the early origin of this insightful comment, this end has yet to be successfully achieved. Since the beginning of the century a plethora of trypanocidal drugs have been developed and tested against experimental trypanosome infections; however, only a few have survived trial in natural infections of man or domestic animals and prolonged use in the field. Drugs routinely used to treat trypanosomiasis were developed prior to the 1950's [Williamson, 1970], and only one new trypanocidal drug, eflornithine, has been introduced since that time.

The trypanocidal drugs outlined here are the ones currently in use and can be divided into two groups: (1) those which are of value only in the early stages of the disease before central nervous system involvement, and (2) those which are effective in advanced infections. Drugs contained within the first group are unable to cross from the blood into the cerebrospinal fluid and therefore are only effective in eliminating trypanosomes from the bloodstream and rendering the animal or human non-infective. The second group of drugs can successfully penetrate the cerebrospinal fluid and are thus able to cure the disease in its late stage [Apted, 1970]. Many of these drugs, especially those effective in the late stages of disease, induce toxic side effects and there have been reports of trypanosome populations exhibiting drug resistance both experimentally and in the field [Apted, 1970; WHO, 1980]. In all cases the precise mode of action is unknown [Williamson, 1970], although the majority of drugs appear to target in some way the trypanosome glycolytic pathway (discussed further in Chapter 1). Treatment regimes are currently of a prolonged nature (usually in the range of 30 days) and generally require hospitalization [WHO, 1980].

(i) Drugs Effective in the Early Stages of Infection

Pentamidine

Pentamidine is generally effective in treating Gambian sleeping sickness (caused by *T.b. gambiense*), although reports of field isolates of *T.b. gambiense* demonstrating resistance to pentamidine are increasing [Kayembe and Wéry, 1972; Dukes, 1984; WHO, 1991]. Resistance to pentamidine in Rhodesian sleeping sickness (caused by *T.b. rhodesiense*) is widespread, and thus this drug is rarely used to treat this form of the disease [Bacchi et al., 1990]. Toxicity associated with administration of this drug includes the induction of diabetes in certain individuals [Collomb et al., 1956]. One advantage to pentamidine use is its shorter treatment course, a mere 7 to 10 days rather than the routine 30 day treatment observed with other drugs. An added benefit of pentamidine is its potential for use as a mass prophylactic agent for Gambian sleeping sickness; a single intramuscular injection of pentamidine can provide protection for up to six months against *T.b. gambiense* strains that have not become drug resistant [Apted, 1970]. Although the mode of action of pentamidine remains unknown [Berger et al., 1995], various targets have been suggested, including S-adenosyl-L-methionine decarboxylase [Bitonti, 1986], mitochondrial topoisomerase II [Shapiro and Englund, 1990; Shapiro, 1993], mitochondrial membrane potential [Vercesi and Docampo, 1992],

dihydrofolate reductase [Waalkes and Makulu, 1976], thymidylate synthetase [Kaplan and Myers, 1977], calcium transport [Benaim et al., 1993] and lysine-arginine transport [Gutteridge, 1969]. This protracted list of suspected targets exemplifies the complex mechanisms of drug action and demonstrates our minimal understanding of these mechanisms.

Berenil

Berenil has been used for decades in the treatment of veterinary trypanosomiasis [Apted, 1970] and has more recently been used for treating both the Gambian [Hutchinson and Watson, 1962] and Rhodesian [Apted, 1970] forms of sleeping sickness. Limited toxicity and resistance have been observed with use of Berenil, most likely due to the rapid metabolization of this drug and excretion of its degradation products, usually within twenty-four hours of treatment [Apted, 1970]. This rapid metabolism has its drawbacks, however, as it necessitates a rigorous and thorough treatment regime to ensure drug success. Once again the mechanism of action for Berenil is unknown, although it is believed to interfere with glycolysis and may also affect the targets listed for pentamidine, as the two drugs belong to the same class of diamidine trypanocides [Apted, 1970].

Suramin

Suramin represents the most widely used drug for treating early stages of trypanosomiasis [WHO, 1991]. This drug is equally effective in treating both Gambian and Rhodesian forms of the disease and is also successful in treatment of veterinary trypanosomiasis. A broad range of toxic reactions have been observed with Suramin treatment, including vomiting, shock, collapse, conjunctivitis, stomatitis and kidney damage. Although the majority of these reactions are mild, fatalities resulting from drug therapy have been reported. Suramin possesses some prophylactic action as it forms a complex with plasma protein and is excreted very slowly, thus protecting from overt infection for up to three months [Apted, 1970]. The mode of action of Suramin has been the most well characterized of all the trypanocidal drugs; it has been shown to specifically inhibit a variety of glycolytic enzymes [Fairlamb and Bowman, 1980; Willson et al., 1993] and in this way interferes with trypanosome glycolysis (discussed in detail in Chapter 1).

(ii) Drugs Effective in the Later Stages of Infection

Tryparsamide

For decades tryparsamide was the most useful drug available for the treatment of late stage Gambian sleeping sickness. This drug is of no value in the Rhodesian disease, as all *T.b. rhodesiense* strains exhibit resistance. Drug resistance has also been noted among an increasing number of *T.b. gambiense* strains and this finding, combined with the long course of treatment required for this drug (approximately three months) and its potential toxicity, have limited its use in recent years. However, tryparsamide is still used in remote regions of Africa where there is minimal medical supervision and where there is a long tradition of such treatment [Apted, 1970; WHO, 1980]. Tryparsamide is a pentavalent aromatic arsenical, and although much less toxic than its parental compound arsenite, the list of side effects attributed to use of this drug is daunting. These include nausea, vomiting, diarrhea, dermatitis and permanent damage to the optic nerve. In some instances reactions to the drug are extremely severe, resulting in shock, convulsions, induction of a comatose state and death [Apted, 1970]. The suspected target for all arsenical derivatives, including tryparsamide and the melaminyl drugs (described below), is once again trypanosome glycolysis, specifically inhibition of the enzyme pyruvate kinase [Flynn and Bowman, 1974].

The Melaminyl Drugs

The severe toxicity and high incidence of resistance associated with use of tryparsamide prompted the development of a new class of trypanocidal drugs, the melaminyl drugs. This group of arsenical derivatives includes the drugs melarsoprol, melarsonyl potassium and melarsen. Melarsoprol is a trivalent arsenical that has been by far the most widely used drug for treating late stage trypanosome infection. This drug has multiple advantages over tryparsamide, including its effectiveness against both the Gambian and the Rhodesian disease, a treatment course of only two to four weeks and few incidences of drug resistance. However, administration of melarsoprol requires close medical supervision for early detection of toxic drug reactions. Toxicity includes fever, chest and abdominal pains and damage to the nervous system. The latter toxic reaction can range from reactive encephalopathy, which is relatively common but from which patients often recover, to the rare but invariably fatal haemorrhagic encephalopathy [Apted, 1970; Haller et al., 1986].

Melarsonyl potassium is a water-soluble analogue of melarsoprol. This drug is advantageous in that it can be administered by intramuscular or subcutaneous injection rather than the more inconvenient intravenous injections required for melarsoprol treatment. However, melarsonyl potassium is ineffective against *T.b. rhodesiense* infections and possesses all of the toxicity associated with melarsoprol. Melarsen is a pentavalent arsenical and was the first of the melaminyl drugs to be introduced. This drug is rarely used due to its high cost and long treatment course (up to three months); however, as for tryparsamide it is useful in remote areas of Africa since it can be administered by trained staff in the field without direct medical supervision. It is effective against both the Gambian and the Rhodesian disease and exhibits toxicity similar to that of melarsoprol [Apted, 1970].

Nitrofurazone

Nitrofurazone is a 5-nitro-substituted furan derivative that has proved favorable in the treatment of Gambian sleeping sickness but is less effective in the treatment of Rhodesian sleeping sickness. Nevertheless, use of this drug is beneficial as a last resort in the treatment of *T.b. rhodesiense* infections that are resistant to the melaminyl drugs. The mode of action of this drug is unknown, and toxic reactions such as polyneuropathy and acute hemolytic anemia are common [Apted, 1970].

Eflornithine

The drug eflornithine (α-difluoromethylornithine) is an irreversible inhibitor of ornithine decarboxylase [Mamont et al., 1978] that has recently been used successfully to treat terminal *T.b. gambiense* infections in patients considered refractory to other trypanocidal drugs [Schechter and Sjoerdsma, 1986]. Inhibition of ornithine decarboxylase by α-difluoromethylornithine results in inhibition of protein and nucleic acid synthesis [Sjoerdsma and Schechter, 1984; Bacchi et al., 1980]. Although this drug uniformly inhibits ornithine decarboxylase in both trypanosomes and mammalian cells, trypanosomes are approximately 100 times more sensitive [Mamont et al., 1978] and thus by closely monitoring the dosage of eflornithine the toxicity to mammalian cells can be minimized. However, this drug is thought to have limited potential: after only ten years of use in the field it is already exhibiting extensive resistance, it has minimal effectiveness against *T.b. rhodesiense* infections and it requires intravenous administration with hospitalization of the patient [Bellofatto et al., 1987]. Fantastic claims for this drug by the

World Health Organization [WHO, 1991] proclaiming it the "resurrection drug" appear to be premature and overblown.

(iii) New Drug Development

In the last four decades only one new drug, the relatively inefficient eflornithine, has been introduced for the treatment of African trypanosomiasis. The current focus of trypanocidal drug development is specific targeting of the trypanosome glycolytic pathway, with particular emphasis on the enzyme glyceraldehyde 3-phosphate dehydrogenase as the three-dimensional crystal structure has been determined for this enzyme [Verlinde et al., 1994; Willson et al., 1994]. However, the trypanosomal glycolytic enzymes thus far characterized, including glyceraldehyde 3-phosphate dehydrogenase, possess a high degree of sequence conservation of active site residues [Michels et al., 1986; Swinkels et al., 1986] and exhibit 45-58% overall sequence identity [Swinkels et al., 1986] with their mammalian counterparts (discussed in detail in Chapter 1). This has generally impeded the design of trypanosome-specific drugs that do not adversely affect the mammalian host. The enzyme described in Chapter 1 of this thesis is a potential candidate for rational drug design which may offer a new approach to treatment.

An additional recent focus of trypanocidal drug development is the effect of the phenothiazine neuroleptic drug, thioridazine. *In vitro* investigations on *T. brucei* BSF have demonstrated that this drug induces rapid morphological changes, including inhibition of cell motility, reorganization of the microtubular membrane skeleton with resultant alteration in cell shape, damage to nuclear and cytoplasmic membranes and release of VSG from the surface membrane [Page and Lagnado, 1995]. The chemotherapeutic potential of thioridazine *in vivo* awaits further study.

The inevitable fatality of advanced trypanosomiasis, combined with the extreme toxicity of the drugs effective in the later stages of infection, stresses the importance of early disease diagnosis. In addition, the currently available sub-optimal treatment regimes for trypanosomiasis emphasizes the urgent need for development of efficient, inexpensive and simply-administered trypanocidal drugs that exhibit minimal toxicity to the host and which have a precisely defined mode of action .

3.3 Vaccine Prospects

Apart from mechanical control measures implemented to minimize contact with the tsetse fly vector and the short-term protection exhibited by some of the previously described drugs, no methods for disease prophylaxis are currently available for trypanosomiasis. This is by no means an exception within the world of parasites; despite the multitude of human parasitic diseases not a single vaccine has been developed.

The occurrence of antigenic variation in African trypanosomes precludes vaccination with lethally irradiated trypanosomes, as such an immunization protocol would only induce protection against trypanosomes expressing the same VSG type. However, the limited number of VATs in the trypanosome metacyclic repertoire (see above) [Turner et al., 1988] suggests the possibility of vaccinating with a cocktail of metacyclic VSG antigens. Two major problems are associated with such a vaccine. First, the number of serodemes circulating in some geographical regions may be very high, and therefore this would increase the complexity required for the vaccine [Vickerman et al., 1993]. Second, the M-VAT repertoire of a given serodeme changes with time as a result of mutation and/or genetic recombination [Turner et al., 1991]. This is exemplified by a case study performed on the constancy of M-VAT expression in the Lake Victoria *T.b. rhodesiense* serodeme, which does not seem to have been maintained over a period of twenty years [Barry et al., 1983].

The limited possibilities for development of a VSG-based vaccine have prompted the search for invariant molecules located on the trypanosome cell surface as possible antigens for immunoprophylaxis. Three groups of such molecules would have vaccine potential: (1) growth factor receptors, which have been demonstrated to be largely localized in the flagellar pocket [Olecnick et al., 1988]; (2) other invariant surface molecules found on trypanosome BSF; and (3) invariant surface molecules found on PCF trypanosomes. The latter group of molecules would be useful in a transmission blocking vaccine, in which vaccination of a host would only serve to prevent infection of the tsetse fly vector and subsequent spread of infections to new mammalian hosts. This type of vaccination protocol would be applicable for cattle trypanosomiasis and would presumably have to be confined to restricted animal access areas where cattle have little interaction with wild reservoir hosts [Vickerman et al., 1993].

4. Cell Surface Molecules

The immunodominance and preponderance of the VSG and procyclin surface molecules on bloodstream and procyclic trypanosomes, respectively, have hampered the identification of underlying, less abundant surface molecules by conventional immunological or protein microchemical techniques. Surface iodination of bloodstream trypanosomes results in the incorporation of label into VSG alone due to its inherent shielding effect on underlying surface molecules [Mancini et al. 1982]. In contrast, more than 25 different molecules incorporate label upon surface iodination of PCF trypanosomes [Gardiner et al., 1983], thus demonstrating that there are numerous molecules in the trypanosome membrane, at least in the procyclic life cycle stage. Identification of invariant cell surface molecules underlying the VSG and procyclin coats has required the application of novel and imaginative protein identification techniques. In recent years many such putative invariant trypanosome cell surface molecules have been identified, although relatively few have been conclusively demonstrated to be present on the cell surface. The molecules for which there is compelling evidence for their cell surface disposition and for which nucleic acid and/or protein sequence information has been obtained are individually addressed in this section. The current status of knowledge on the remaining putative invariant cell surface molecules, which have been collectively less well characterized, is summarized in Tables 1, 2 and 3. These putative cell surface molecules have been divided into three groups: (1) those expressed exclusively on BSF trypanosomes; (2) those expressed exclusively on PCF trypanosomes; and (3) those expressed on both BSF and PCF trypanosomes.

(i) VSG

VSGs from different VATs have molecular weights ranging from 46 kDa (*T. vivax* [Gardiner et al., 1987]) to 65 kDa (*T. brucei* [Cross, 1975]) and in *T. brucei* spp. are expressed on the cell surface at a density of approximately 10^7 molecules per cell [Turner et al., 1985; Cross, 1990]. *T. brucei* VSGs have been shown to contain between 7 and 17% carbohydrate [Johnson and Cross, 1977]. The C-terminal domain of approximately 120 amino acids shows some degree of sequence conservation between different VSGs, but the NH_2-terminal domains are diverse in sequence and thus account for their varying antigenicity. Even here there is some sequence conservation however [Olafson et al., 1984]. The C-terminal region contains a hydrophobic tail that is replaced by a glycosylphosphatidylinositol (GPI) anchor that is sensitive to cleavage by phospholipase

co-purification with plasma membranes and immunoprecipitation from detergent extracts of ^{125}I-labeled parasites using antiserum raised against purified plasma membranes or using antiserum from parasitemic rabbits [Jackson et al., 1993b].

The genes encoding ISG65 and ISG75 were isolated by screening a *T. brucei* cDNA expression library with an antiserum prepared against the concanavalin A-binding protein preparation described above. The nucleotide sequence of the isolated genes suggests no relationships to other known genes and predicts polypeptides with NH_2-terminal signal sequences, hydrophilic extracellular domains, single trans-membrane α-helices and short cytoplasmic domains. Immunofluorescence analysis demonstrated that ISG65 and ISG75 are distributed over the entire surface of the parasite [Ziegelbauer et al., 1992].

(iv) Transferrin-Binding Protein

A transferrin-binding protein of Mr 42 kDa was purified from *T.b. brucei* BSF by affinity chromatography on human transferrin-coupled Sepharose [Schell et al., 1991]. The C-terminus of this protein is modified by a phospholipase C-sensitive GPI anchor. The transferrin-binding protein was expressed only in BSF trypanosomes at a copy number of approximately 1000 molecules per cell and is encoded by expression site-associated gene 6 (ESAG 6). The ESAGs 6 and 7 exhibit substantial sequence similarity, suggesting ESAG 7 may also encode a transferrin-binding protein. The predicted subcellular location for the transferrin-binding protein is the flagellar pocket [Schell et al., 1991], which is formed by an invagination of the plasma membrane at the emerging flagellum. The flagellar pocket is believed to be an important site for several processes including nutrient uptake [McLaughlin, 1987], recycling of membrane components [Frevert and Reinwald, 1988; Webster and Grab, 1988] and secretion [Steiger, 1971; Steiger, 1973; Vickerman, 1969]. Within this locale the transferrin-binding protein would bind transferrin and the complex would be rapidly internalized [Schell et al., 1991].

(v) Low Density Lipoprotein Receptor

T. brucei spp. parasites demonstrate receptor-mediated endocytosis of low density lipoprotein (LDL) particles from mammalian hosts. The LDL receptor responsible for this phenomenon has been purified to near homogeneity from BSF and PCF of *T.b. brucei* by

C. VSG appears to function as a barrier, preventing host macromolecules (i.e. antibodies, complement) from reaching the plasma membrane [Vickerman et al., 1993].

There are estimated to be more than 1000 different VSG genes in the *T. brucei* genome [Van der Ploeg et al., 1982]. Only one VSG gene is expressed at a time, and the active copy is always present at the 3' end of a large, multigenic and telomeric transcription unit [Johnson et al., 1987; Kooter et al., 1987; Pays et al., 1989]. The genes belonging to this unit are referred to as expression site-associated genes (ESAGs) [Cully et al., 1985]. At least 7 ESAGs have thus far been identified [Smiley et al., 1990] and their expression is coordinated with that of the VSG gene [Cully et al., 1985; Kooter et al., 1987; Alexandre et al., 1988; Pays et al., 1989]. The VSG molecules, their encoding genes and mechanisms of gene expression have been reviewed elsewhere [Borst and Cross, 1982; Boothroyd, 1985; Donelson and Rice-Ficht, 1985a; Donelson and Turner, 1985b; Borst, 1986; Vickerman et al., 1993].

(ii) The Procyclins

The procyclin molecules have an apparent molecular mass in SDS-PAGE of between 30 and 40 kDa [Richardson et al., 1988], are expressed on the cell surface at approximately 6×10^6 molecules per cell and are highly negatively charged [Clayton and Mowatt, 1989]. The procyclins of *T. brucei* spp. are characterized by a 31-amino acid NH_2-terminal domain containing an N-linked glycosylation site followed by an extensive glutamic acid-proline repeat that runs throughout approximately 40% of their primary structure [Roditi and Pearson, 1990]. The procyclin glycoproteins possess a glycolipid membrane anchor that differs from that of VSG molecules and is insensitive to cleavage by phospholipase C [Field et al., 1991]. The *T. brucei* spp. procyclins are glycosylated at the one N-linked site [Richardson et al., 1988] and at the membrane anchor, where a complex, highly sialated structure exists [Ferguson et al., 1993]. The primary sequence of the molecule indicates that it probably forms an extended, rigid, tertiary structure with its $(Asp\text{-}Pro)_2(Gln\text{-}Pro)_{22\text{-}29}$ repeats forming a cylindrical structure [Roditi et al., 1989]. The *T. brucei* spp. procyclin genes exist in four to seven copies per haploid genome, distributed in two or three clusters of two to three tandemly linked genes, depending on the strain [Mowatt and Clayton, 1987; Mowatt and Clayton, 1988; Koenig et al., 1989; Mowatt et al., 1989]. All these genes appear to be transcribed [Koenig et al., 1989; Mowatt et al., 1989]. The procyclin genes have been discussed in detail elsewhere [Hehl and Roditi, 1994].

In *T. congolense* the major surface molecules of procyclics show no sequence similarity to the *T. brucei* spp. procyclins yet are acidic and immunodominant glycoproteins that are expressed with the same kinetics during differentiation from bloodstream to procyclic forms and form a surface coat [Beecroft et al., 1993; Bayne et al., 1993]. Like the *T. brucei* spp. procyclins, they are lipid anchored, show a subgenus specific distribution, and probably exhibit an extended polyanionic structure. Curiously, the *T. congolense* molecules show no sites for N-linked glycosylation. They do, however, have several sites for *O*-glycosylation and are heavily glycosylated [Beecroft et al., 1993]. Thus, the *T. congolense* molecules (named GARP, for glutamic acid/alanine-rich protein) are probably analogs, not homologs of the *T. brucei* spp. procyclin (PARP) molecules.

Although the function of the procyclins is unknown, these glycoproteins are proposed to protect the parasite from proteases in the tsetse fly midgut and/or play a role in determining tropism within the vector [Roditi and Pearson, 1990; Stebeck and Pearson, 1994].

(iii) Invariant Surface Glycoproteins

Two invariant surface glycoproteins (ISGs) have been isolated from *T.b. brucei* BSF and characterized at the molecular level. These are ISG65 (M_r 65 kDa) [Ziegelbauer and Overath, 1992; Ziegelbauer et al., 1992; Jackson et al., 1993b] and ISG75 (M_r 75 kDa) [Ziegelbauer and Overath, 1992; Ziegelbauer et al., 1992; Ziegelbauer et al., 1995]. These ISGs were identified on the surface of *T.b. brucei* BSF by a combination of cell surface biotinylation, release of VSG from the cell membrane via use of the endogenous GPI-specific phospholipase C, and subsequent Triton X-114 phase separation and precipitation with streptavidin and concanavalin A coupled to solid supports [Ziegelbauer and Overath, 1992]. In an alternative method ISG65 was also identified by direct cell surface radioiodination of *T.b. brucei* BSF [Jackson et al., 1993b]. These polypeptides were detected in trypanosomes expressing different VSGs and are thus considered to be invariant [Ziegelbauer and Overath, 1992; Jackson et al., 1993b]. Neither ISG65 nor ISG75 could be detected in PCF trypanosomes [Ziegelbauer and Overath, 1992]. Both ISG65 and ISG75 were determined to be encoded by a multi-gene family [Ziegelbauer et al., 1995] and expressed on the cell surface at 7×10^4 and 5×10^4 copies per cell, respectively [Ziegelbauer et al., 1992; Overath et al., 1994]. Protein microchemical evidence supporting the designation of ISG65 as an integral membrane protein includes

affinity chromatography on human LDL-coupled Sepharose [Coppens et al., 1988; Coppens et al., 1991]. This molecule has a molecular mass of 145 kDa and is glycosylated [Coppens et al., 1991]. Monospecific antibodies were raised against the trypanosome LDL receptor and used in immunofluorescence to localize the receptor to the flagellar pocket. LDL binding experiments estimated trypanosomes express 52,000 copies of low-affinity receptors and 1800 copies of high-affinity receptors [Coppens et al., 1988]. In addition to protein microchemical characterization, the LDL receptor has also been characterized at the molecular level by Lee et al. [1990]. These investigators characterized a cDNA encoding a cysteine-rich, acidic integral membrane protein that showed striking sequence similarity to the human LDL receptor. Immunofluorescence studies showed the translated product was localized to the flagellar pocket, and sequence analyses performed on the translated protein revealed it contained a signal peptide, a transmembrane domain and a large extracellular domain.

(vi) Adenylate Cyclases

Two *T. brucei* spp. genes have been identified that encode proteins exhibiting sequence similarities to eukaryotic adenylate cyclase. Expression of one gene is restricted to BSF trypanosomes; this gene is contained within the ESAG family (ESAG 4) and encodes a protein of 150 kDa. A second gene related to ESAG 4, termed the gene related to expression site-associated gene 4.1 (GRESAG 4.1), is expressed in both BSF and PCF trypanosomes and encodes a protein of a similar molecular mass. These genes differ primarily in the 5' region of the coding sequence, which is presumed to encode a large extracellular domain [Paindavoine et al., 1992]. Hydropathy analysis revealed in each case two probable membrane-spanning segments flanking this NH_2-terminal extracellular domain [Alexandre et al., 1990]. Immunofluorescence analysis using polyclonal antiserum raised against the recombinant ESAG 4 product showed specific fluorescence localized to the flagellar membrane in both PCF and BSF trypanosomes, and immunoblotting demonstrated that trypanosome membrane preparations from the two life cycle stages contained immunoreactive proteins of approximately 150 kDa. In addition, the recombinant products of ESAG 4 and GRESAG 4.1 can complement a *Saccharomyces cerevisiae* mutant for adenylate cyclase. The adenylate cyclases of the two life cycle stages appear to differ in their sensitivity to calcium, as the ESAG 4 cyclase demonstrates activation and the GRESAG 4.1 cyclase exhibits inhibition in the presence of calcium. Although not formally investigated, immunofluorescence analysis predicts

the adenylate cyclase cell copy number to be quite low for both PCF and BSF trypanosomes [Paindavoine et al., 1992].

(vii) Glucose Transporters

Bringaud and Baltz [1992] identified a developmentally regulated *T.b. brucei* cDNA clone by expression library screening. This clone encoded a putative membrane protein of 56.5 kDa that showed significant sequence similarity to the erythrocyte glucose transporter and was found to be expressed at a much higher level in the BSF parasites. Previous studies performed by Parsons and Nielsen [1990] and Seyfang and Duszenko [1991] had demonstrated that BSF trypanosomes employ facilitated diffusion for glucose uptake, while PCF trypanosomes actively transport glucose, thus suggesting the presence of different glucose transporters in the two life cycle stages. Subsequent investigations have shown that stage-specific transporters are encoded by two groups of genes exhibiting 80% sequence similarity, designated trypanosome hexose transporter (THT)1 (six copies) and THT2 (five copies) [Bringaud and Baltz, 1993]. The plasma membrane disposition of the BSF trypanosome glucose transporter was verified by purification from the trypanosome cell membrane, reconstitution into liposomes and subsequent analysis of glucose transport [Seyfang and Duszenko, 1993].

(viii) Procyclic Stage Surface Antigen (PSSA)-2

This surface protein was identified through transient expression of *T.b. brucei* cDNA libraries in mammalian COS cells and subsequent 'panning' for surface antigen cDNAs with a rabbit polyclonal antiserum raised against PCF trypanosomes. The PSSA-2 cDNA selected via this method encoded a novel 40 kDa antigen with the features of a typical transmembrane protein and containing an unusual cytoplasmic tail composed of a proline-rich tandem repeat. Immunofluorescence analysis using the polyclonal antiserum showed COS cells expressing PSSA-2 exhibited specific surface fluorescence. The PSSA-2 transcript was only observed in PCF trypanosomes. PSSA-2 is estimated to be expressed at 3×10^5 molecules per cell, and southern blot analysis demonstrated it is encoded by more than one gene copy [Jackson et al., 1993a].

Other putative trypanosome surface molecules are not so well characterized and are summarized in the following three tables.

Table 1 Putative invariant surface molecules expressed exclusively on bloodstream form trypanosomes.

Prospective cell surface molecule	Isolation/ characterization method	Gene(s)	Molecular mass (kDa)	Copy number per cell	Putative subcellular localization	Putative function	Reference(s)
ISG60	-surface biotinylation	?	60	50,000	surface	?	Ziegelbauer and Overath, 1992
ISG64	-surface iodination	?	64	?	surface	?	Jackson et al., 1993b
proteins encoded by ESAGs	-gene cloning	ESAGs	various	?	surface	nutrient uptake or inhibition of host immune response	Cully et al., 1985 Cully et al., 1986 Kooter et al., 1987 Pays et al., 1989
acid phosphatase	-enzyme analysis -cell fractionation	?	?	?	flagellar pocket and surface	release/turnover of VSG	McLaughlin, 1986
Ca^{2+}-ATPase	-enzyme analysis -cell fractionation	?	?	?	surface	regulating intracellular Ca^{2+}	McLaughlin, 1985a
M_r 84-140 kDa glycoproteins	-affinity chromatography -immunofluorescence	?	84-140	?	flagellar pocket	?	Brickman and Balber, 1993
miscellaneous glycoproteins	-metabolic labeling -surface iodination -affinity chromatography	?	76, 86, 92-100 42, 130	?	surface (extrinsic) surface (intrinsic)	?	Mancini et al., 1982

Table 2 Putative invariant surface molecules expressed exclusively on procyclic form trypanosomes.

Prospective cell surface molecule	Isolation/ characterization method	Gene(s)	Molecular mass (kDa)	Copy number per cell	Putative subcellular localization	Putative function	Reference(s)
sialidase	-gel filtration -ion-exchange chromatography -enzyme analysis	?	67	?	surface (integral membrane protein)	release of sialic acids	Engstler et al., 1992
M_r 88 kDa glycoprotein	-surface iodination -lectin binding -cytoskeletal preparations	?	88	?	surface (transmembrane protein?)	linkage of the cytoskeleton to the membrane	Gardiner et al., 1983 Woods et al., 1989a
flagellar pocket antigens	-immunofluorescence microscopy	?	?	?	flagellar pocket	?	Woods et al., 1989b
procyclin associated genes							
-PAG 1	-gene cloning	1	40	?	surface	receptor?	Koenig-Martin et al., 1992
-GRESAG 2	-gene cloning	1	45	?	surface	?	Berberof et al., 1991
miscellaneous glycoproteins	-surface biotinylation	?	81.5 59 38-42	?	surface	?	Mutharia and Steele, 1995

Table 3 Putative invariant surface molecules expressed on both bloodstream and procyclic form trypanosomes.

Prospective cell surface molecule	Isolation/ characterization method	Gene(s)	Molecular mass (kDa)	Copy number per cell	Putative subcellular localization	Putative function	Reference(s)
epidermal growth factor receptor	-immunofluorescence microscopy	?	135	?	surface	binds mammalian host factors involved in parasite growth and differentiation	Hide et al., 1989 Hide et al., 1990
3'-nucleotidase	-enzyme analysis	?	?	?	surface	acquisition of purines	Gottlieb et al., 1986 Gottlieb, 1989

5. Thesis Rationale and Research Objectives

Despite more than a century of research, treatment regimes for African sleeping sickness are sub-optimal and tsetse fly avoidance currently represents the only means of disease prophylaxis. The need for trypanosomiasis control has prompted a quest for the development of improved chemotherapeutic agents for disease mediation and for the identification of potential vaccine candidates for disease prevention. Findings presented in this thesis may contribute to this endeavor.

The original focus of this thesis was the identification, isolation and molecular characterization of novel molecules present on the cell surface of African trypanosomes, an undertaking prompted by the paucity of information on well-characterized surface molecules on the parasite membrane. The plasma membrane represents the interface between the trypanosome and its mammalian host and tsetse fly vector, and thus characterization of its surface molecules could lead to the identification of structurally or functionally essential molecules, some of which may participate in parasite-host and/or parasite/vector interactions. In addition to providing a more integrated understanding of the trypanosome membrane, these studies were initiated to identify potential chemotherapy or vaccination targets for control of trypanosomiasis.

My research revealed several molecules associated with the trypanosome cell membrane. One of these, described in Chapter 1, was clearly membrane associated although inexplicably so, and was characterized as glycerol 3-phosphate dehydrogenase. A second molecule, an 11 kDa trypanosome kinetoplastid membrane protein, was shown to be associated with cell membranes and is described in Chapter 2.

As discussed in the general introduction, glycolysis in African trypanosomes occurs within membrane-bounded organelles called glycosomes. Glycerol 3-phosphate dehydrogenase is also glycosomally localized, and the activity of this enzyme is intimately associated with glycolysis. Therefore, in order to gain an integrated understanding of the functional relevance of glycerol 3-phosphate dehydrogenase to African trypanosomes, the introduction to Chapter 1 focuses upon glycolysis within these kinetoplastid parasites.

6. Contributors to Work Presented in this Thesis:

I thank the following individuals for their contribution to work presented in this thesis. In addition to this general description of work contributed by the respective scientists, specific descriptions are provided throughout the thesis.

a) Dr. Isabel Roditi and Dr. Erik Vassella (Department of General Microbiology, University of Bern, Bern, Switzerland) aided in our initial polymerase chain reaction amplification of the cDNA encoding the glycerol 3-phosphate dehydrogenase.

b) Dr. Ute Frevert and Gabrielle Pradel (Department of Medical and Molecular Parasitology, New York University Medical Center, New York, New York, USA) performed the immunogold electron microscopy for localization of the glycerol 3-phosphate dehydrogenase.

c) Roseanne McIndoe (Department of Pathology, University of British Columbia, Vancouver, British Columbia, Canada) helped with the confocal laser scanning immunofluorescence microscopy to aid in localization of the kinetoplastid membrane protein-11.

d) Dr. Thomas P. Mommsen and Glenn Cooper (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) performed the glycerol 3-phosphate dehydrogenase enzyme assays.

e) Gerald Baron (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) performed the endoproteinase Lys-C digestion of the trypanosome 11 kDa protein to obtain internal amino acid sequence information.

f) Robert P. Beecroft (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) aided in generating the glycerol 3-phosphate dehydrogenase-specific monoclonal antibodies and participated in the immunological characterization and purification of the trypanosome kinetoplastid membrane protein-11.

g) Corinna Tuckey (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) isolated the bacteriophage DNA

encoding a 92 amino acid protein identified by screening of a cDNA expression library with the kinetoplastid membrane protein-11-specific monoclonal antibodies.

h) Michael Bridge (Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada) aided in characterizing the glycerol 3-phosphate dehydrogenase and kinetoplastid membrane protein-11 P1 clones in preparation for knockout mutagenesis.

CHAPTER 1: Molecular Characterization of the NAD^+-Dependent Glycerol 3-Phosphate Dehydrogenase from *Trypanosoma brucei rhodesiense*

INTRODUCTION

One of the unique characteristics of the protozoan hemoflagellates belonging to the order Kinetoplastida is their unusual and complex compartmentalization of carbohydrate metabolism. Although early reports eluded to the presence of such compartmentalization [Ryley, 1962; Risby et al., 1969; Risby and Seed, 1969; Reynolds, 1975], its true nature was not understood until 1977 when nine enzymes were shown to be associated with membrane-bounded organelles in *T. brucei*. Seven of these enzymes were found to be involved in glycolysis (hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase), while two were found to be involved in glycerol metabolism (glycerol kinase and glycerol 3-phosphate dehydrogenase) [Opperdoes and Borst, 1977]. Subsequent investigations have revealed that these organelles are found throughout kinetoplastid parasites [Taylor et al., 1979; Cannata et al., 1982; Hart and Opperdoes, 1984] and that they contain additional enzymes involved in a diverse range of metabolic processes, including carbon dioxide fixation, pyrimidine biosynthesis, ether-lipid biosynthesis and purine salvage [Opperdoes, 1987]. These organelles are similar to the microbody class of eukaryotic organelles comprising the peroxisomes of plant and animal cells and the glyoxysomes of plant cells [Opperdoes and Borst, 1977; deDuve, 1982]. The compartmentalization of glycolytic enzymes within these microbodies, as opposed to other eukaryotic cells where glycolytic enzymes have a cytosolic locale [Hart et al., 1984], prompted the designation of these organelles as glycosomes [Opperdoes and Borst, 1977].

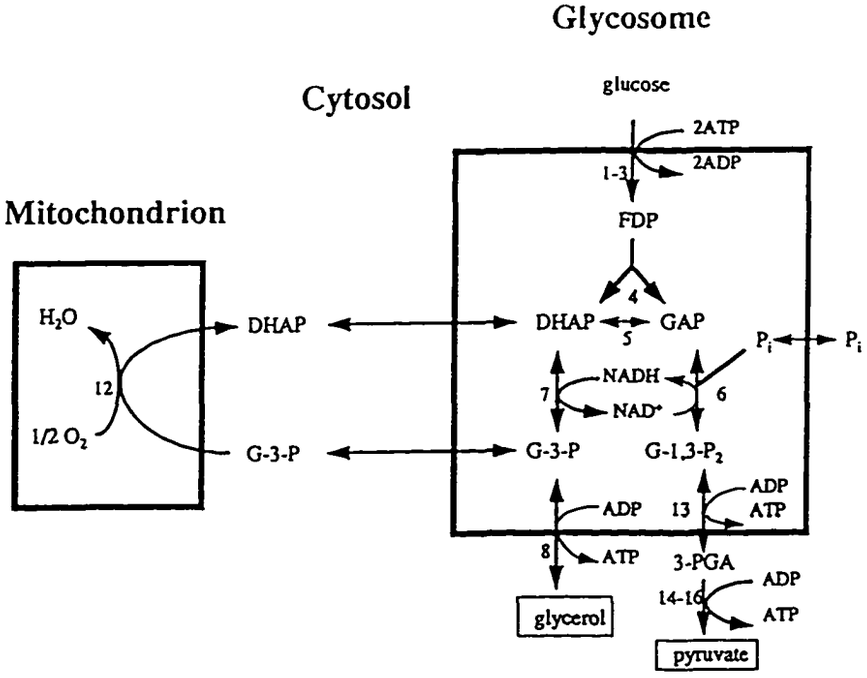
Glycosomes have been best studied in *T.b. brucei*, undoubtedly due to the central relevance of these organelles to energy generation and thus parasite survival. Morphometric analyses have shown glycosomes to be homogeneous in size, with an average diameter of 0.27 μm [Opperdoes, 1984]. Trypanosomes contain an average of 240 glycosomes [Opperdoes, 1987], which represents between 4.3% [Opperdoes, 1984] and 8% [Böhringer and Hecker, 1975] of the total volume and protein content [Opperdoes, 1984; Misset et al., 1986] of the cell irrespective of life cycle stage

[Böhringer and Hecker, 1975; Ghiotto et al., 1979]. In striking contrast, the contribution of the trypanosome mitochondrion to total cellular volume is strictly dependent upon life cycle stage ranging from 5% in bloodstream form parasites to 25% in insect-stage parasites [Opperdoes et al., 1976a; Hart et al., 1984]. Morphological and biochemical mitochondrial variations coincide with the stage-dependent alterations in mitochondrial volume. In the long-slender bloodstream form the single mitochondrion is reduced to a peripheral canal with almost no cristae, cytochromes are absent and the Krebs cycle is nonfunctional [Bowman and Flynn, 1976], while transformation to the short, stumpy bloodstream form is marked by a swelling of the mitochondrial canal, development of tubular cristae and some participation in cellular metabolism [Flynn and Bowman, 1973; Bowman and Flynn, 1976]. Procyclic midgut stage trypanosomes possess cytochromes, a fully functional Krebs cycle and a well-developed mitochondrion with an extensively branched network of prominent cristae [Vickerman, 1965].

This life cycle stage-dependent variation in mitochondrial volume, biochemistry and morphology mirrors the profound changes that occur in carbohydrate metabolism and energy derivation throughout the trypanosome life cycle (Figure 2). Due to the absence of a functional mitochondrion and the lack of polysaccharide or high-energy phosphate reserves, the energy requirements of the long-slender bloodstream form are met entirely by the synthesis of ATP via glycolysis using an exogenous carbohydrate source [Hart et al., 1984]. The glycosome contains enzymes that convert glucose and glycerol to glycerate 3-phosphate, while the enzymes involved in converting glycerate 3-phosphate to pyruvate are cytosolically localized [Misset and Opperdoes, 1987]. The glycolytic flux of carbon through the cell is very high, with 0.08 μmol of glucose being consumed per minute per milligram protein [Fairlamb and Bowman, 1980]. This value is at least 10-fold higher than most cells of the mammalian host and represents one of the highest rates of glucose consumption among eukaryotic cells [Cazzulo, 1992]. Under aerobic conditions two molecules of ATP are synthesized for every molecule of glucose metabolized. Pyruvate is the sole end product of glycolysis under such conditions and is excreted into the host's bloodstream [Clarkson and Brohn, 1976]. Under anaerobic conditions equimolar amounts of pyruvate and glycerol are excreted into the host's bloodstream, with the synthesis of one ATP molecule per glucose molecule metabolized [Opperdoes et al., 1976b; Ryley, 1962]. This net ATP synthesis occurs by way of reversal of the glycerol kinase-catalyzed reaction [Opperdoes et al., 1976b] (refer to Figure 2) and is a prime example of the advantage procured by compartmentalization of glycolysis in trypanosomes.

Figure 2 Pathways of glucose metabolism in (A) bloodstream form trypanosomes and (B) procyclic culture form trypanosomes. For simplicity only an abbreviated metabolic pathway is shown in panel A. End products of aerobic or anaerobic metabolism are enclosed in boxes. The dashed lines indicate enzymes whose presence remains uncertain. Enzyme identities: 1, hexokinase; 2, glucosephosphate isomerase; 3, phosphofructokinase; 4, aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde 3-phosphate dehydrogenase; 7, NAD^+-dependent glycerol 3-phosphate dehydrogenase; 8, glycerol kinase; 9, malate dehydrogenase; 10, adenylate kinase; 11, phosphoenol pyruvate carboxykinase; 12, FAD-dependent glycerol 3-phosphate dehydrogenase/trypanosome alternative oxidase; 13, phosphoglycerate kinase; 14, phosphoglycerate mutase; 15, enolase; 16, pyruvate kinase; 17, aspartate aminotransferase; 18, malic enzyme; 19, alanine aminotransferase; 20, fumarate hydratase; 21a, fumarate reductase; 21b, succinate dehydrogenase; 22, α-oxoglutarate decarboxylase; 23, isocitrate dehydrogenase; 24, aconitase; 25, citrate synthetase; 26, pyruvate dehydrogenase [Opperdoes, 1987].

A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Cytosol
glucose
2ATP
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1-3
Mitochondrion
FDP
4
H_2O
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P_i
12
7
NADH
NAD+
6
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G-3-P
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3-PGA
ADP
14-16
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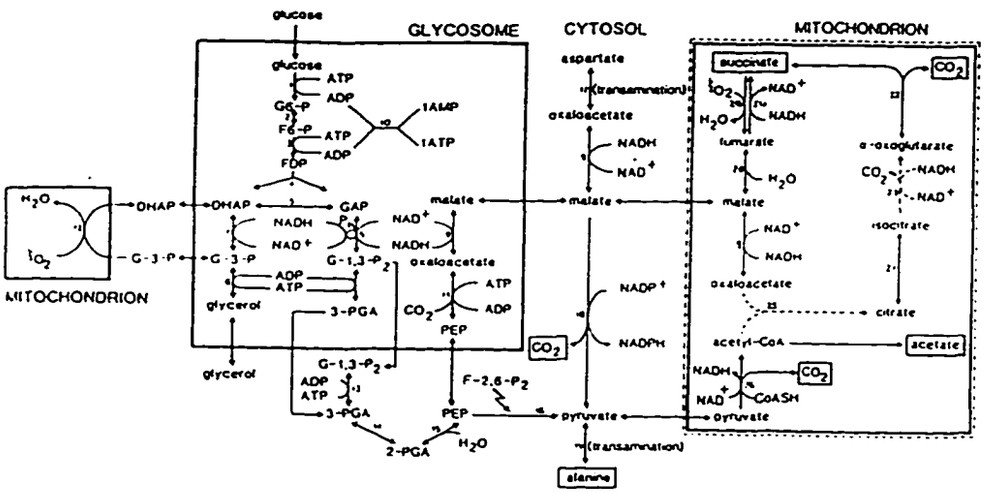
GLYCOSOME
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1ATP
DHAP
GAP
NADH
NAD+
malate
oxaloacetate
glycerol
3-PGA
PEP
pyruvate
succinate
fumarate
citrate
acetyl-CoA
acetate
alanine
aspartate
transamination
CO_2
NADPH
NADP+
CoASH
2-PGA
H_2O
$F\text{-}2,6\text{-}P_2$

After transformation to the procyclic life cycle stage the parasite adapts to the more complex nutritional environment in the insect midgut and utilizes its fully functional mitochondrion to derive energy from the oxidation of amino acids, Krebs cycle intermediates or fatty acids [Hart et al., 1984]. The consumption of glucose occurs at a much reduced rate in this life cycle stage compared to the long-slender bloodstream form, and the end products of metabolism excreted into the host's bloodstream are succinate, acetate and CO_2 [Opperdoes, 1987]. The short-stumpy bloodstream form has partial mitochondrial activity and thus has a metabolism intermediary to the polar extremes of the long slender bloodstream form and procyclic life cycle stage [Flynn and Bowman, 1973; Hart et al., 1984]. The metabolism of the epimastigote and metacyclic life cycle stages has not yet been investigated [Opperdoes, 1987], primarily because of the difficulty of obtaining large enough numbers of the parasites.

Glycolytic systems invariably require an efficient mechanism for reoxidation of the NADH generated through the glyceraldehyde 3-phosphate dehydrogenase reaction to ensure continuation of glycolysis. The procyclic life cycle stage trypanosome utilizes glycosome-localized phosphoenolpyruvate carboxykinase and malate dehydrogenase to convert phosphoenolpyruvate to malate within the glycosome with the concomitant regeneration of NAD^+ (refer to figure 2). This NADH-reoxidation pathway is not used in bloodstream trypanosomes as glycosomes in this life cycle stage are devoid of these two enzymes. Bloodstream forms are also unable to use traditional eukaryotic methods to reoxidize NADH due to the lack of a functional mitochondrion, and thus a cytochrome *c* oxidase, and the lack of lactate dehydrogenase [Kornblatt et al., 1992]. This life cycle stage has instead evolved a unique NAD^+-regenerating glycerol 3-phosphate shuttle between the glycosome and the mitochondrion. Glycosome-produced NADH is used by the glycosomal NAD^+-dependent glycerol 3-phosphate dehydrogenase to reduce dihydroxyacetone phosphate to glycerol 3-phosphate thus regenerating the NAD^+ required for glycolysis. Glycerol 3-phosphate leaves the glycosome and is taken up by the limited mitochondrion where it is reoxidized to dihydroxyacetone phosphate which then returns to the glycosome completing the shuttle [Fairlamb et al., 1977; Opperdoes, 1987; Kornblatt et al., 1992; Barnard et al., 1993]. The net result is that the glycerol 3-phosphate and dihydroxyacetone phosphate are used in only catalytic amounts with virtually all of the triose phosphates produced by aldolase flowing through the glycolytic pathway producing ATP. Within the mitochondrion glycerol 3-phosphate is oxidized to dihydroxyacetone phosphate presumably by an FAD-containing glycerol 3-phosphate

dehydrogenase which also reduces coenzyme Q_9. The abbreviated mitochondrial electron transport chain ends with the trypanosome alternative oxidase which transfers electrons from reduced coenzyme Q_9 to molecular oxygen [Clarkson et al., 1989]. In summary, the glycerol 3-phosphate shuttle transfers electrons to the mitochondrion which transfers them to molecular oxygen without capturing energy in the form of ATP; thus the glycosomal glycerol 3-phosphate dehydrogenase plays a key role in glycolysis under aerobic conditions.

Long-slender bloodstream form parasites have been purported to be able to survive anaerobically for at least 30 minutes [Opperdoes et al., 1976b]. During this anaerobisis the trypanosome alternative oxidase, and thus the glycerol 3-phosphate shuttle, is inoperative. NADH-reoxidation still occurs by way of the NAD^+-dependent glycerol 3-phosphate dehydrogenase-catalyzed reaction; however, the resulting glycerol 3-phosphate, rather than being reoxidized to dihydroxyacetone phosphate as under aerobic conditions, is instead further metabolized by glycerol kinase to glycerol with the concomitant net synthesis of one ATP molecule [Opperdoes, 1987]. Therefore, the functioning of the glycosomal NAD^+-dependent glycerol 3-phosphate dehydrogenase is essential to allow regeneration of NAD^+ and thus continued glycolysis under both aerobic and anaerobic conditions.

The indispensable nature of the NAD^+-dependent glycerol 3-phosphate dehydrogenase to the survival of bloodstream trypanosomes has been further demonstrated by studies of *in situ* parasite inhibition. The inhibition of the glycerol 3-phosphate shuttle observed under anaerobic conditions can be mimicked by the addition of salicylhydroxamic acid (SHAM), a powerful inhibitor of the trypanosome alternative oxidase [Opperdoes et al., 1976b]. In 1959 it was first recognized that this oxidase inhibition was insufficient to induce parasite elimination from the bloodstream of an infected mammalian host [Fulton and Spooner, 1959], a phenomenon easily explained by our current understanding of the ability of trypanosomes to compensate for elimination of oxidase activity by reversal of the glycerol kinase-catalyzed reaction and thus still achieve net ATP synthesis. Studies performed roughly twenty years later, however, demonstrated that concurrent injection of SHAM and glycerol into the bloodstream of an infected mammal effected rapid parasite elimination through inhibition of both aerobic and anaerobic parasite metabolism [Clarkson and Brohn, 1976]. As the NAD^+-dependent glycerol 3-phosphate dehydrogenase enzyme is central to both of these metabolic pathways, these studies demonstrated that development of a specific enzyme inhibitor

would serve to block aerobic and anaerobic metabolism in addition to glycolytic NAD^+-regeneration, effectively crippling the parasite and ensuring elimination from the bloodstream.

An additional role of the NAD^+-dependent glycerol 3-phosphate dehydrogenase in trypanosomes is its involvement in glycerol metabolism. As glycerol is a building block for fatty acid synthesis, conversion of dihydroxyacetone phosphate to glycerol 3-phosphate and subsequently to glycerol by the NAD^+-dependent glycerol 3-phosphate dehydrogenase and glycerol kinase, respectively, is of utmost importance. Remarkably little attention has been focused on this function, but it is nonetheless vital to proper functioning of the parasite.

Previous investigations on the trypanosome NAD^+-dependent glycerol 3-phosphate dehydrogenase have been confined to characterization and glycosomal localization of enzyme activity [Opperdoes and Borst, 1977; Opperdoes et al., 1977; Hart et al., 1984]. Ironically, this was the first enzyme to be localized to the glycosome [Opperdoes et al., 1977] and it is among the last to be characterized at the molecular level. This chapter describes the isolation and molecular characterization of the NAD^+-dependent glycerol 3-phosphate dehydrogenase (GPD; EC 1.1.1.8) from *T.b. rhodesiense*. Considerable emphasis will be given to the method used to identify this enzyme and its potential functional relevance to enzyme activity. The primary structure of the enzyme was determined using a combination of protein microsequencing, polymerase chain reaction amplification of complementary and genomic DNA and DNA sequencing. The trypanosome molecule was expressed in *Escherichia coli* and was found to be enzymatically active, thus confirming the identity of the molecule as an NAD^+-dependent glycerol 3-phosphate dehydrogenase. Immunological analyses using monoclonal antibodies specific for the 38 kDa protein localized the enzyme to glycosomes. Characteristics of the enzyme which may allow rational drug design and the potential of this enzyme as a target for knockout mutagenesis are discussed.

MATERIALS AND METHODS

Parasites. Bloodstream forms (BSF) of *T.b. rhodesiense* EATRO 1989 [Hill et al., 1978] and *T. congolense* IL3000 [Fish et al., 1989] were obtained from Dr. G. Hill (Meharry Medical School, Nashville, TN, USA) and Dr. W. Fish (International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya), respectively. *T.b. rhodesiense* ViTat 1.1 was cloned by micromanipulation from the EATRO 1989 stock [Richardson et al., 1986]. BSF parasites were harvested aseptically from cyclophosphamide-treated [Smith et al., 1982] infected rats by Percoll™ isopycnic centrifugation [Grab and Bwayo, 1983] of whole heparinized blood followed by either lysis of contaminating erythrocytes with 0.83% NH_4Cl in 100mM Tris (pH 7.4) or by purification over a diethylaminoethyl (DEAE)-cellulose column [Lanham and Godfrey, 1970]. Procyclic culture forms (PCF) of *T.b. brucei* 427, *T.b. rhodesiense* ViTat 1.1, *T. congolense* IL3000 and *T. simiae* CP11 were established from the corresponding cloned bloodstream populations [Brun and Schonberger, 1979] and were maintained in culture at 26°C in minimal essential medium (MEM) containing Earle's salts/25 mM HEPES/10% heat-inactivated fetal bovine serum (FBS)/1% non-essential amino acids/2 mM glutamine/60 mM proline/200 μm hypoxanthine/50 μg ml^{-1} gentamycin (procyclic culture medium). *T. congolense* IL3000 PCF, epimastigotes and metacyclics were grown in vitro using culture methods developed at ILRAD [Fish et al., 1989; Bienen et al., 1991]. *Trypanosoma cruzi* Peru strain (WHO reference strain MHOM/PE/00/Peru) was obtained from the American Type Culture Collection (ATTC). Epimastigotes were grown in LIT medium [Castellani et al., 1967] containing 10% FBS. *T. cruzi* Y strain trypomastigotes were obtained from Dr. V. Nussenzweig (NYU, New York, NY, USA) and were grown in Vero cell cultures as described by Schenkman et al. [1991]. Briefly, Vero cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS, penicillin and streptomycin at 37°C in 5% CO_2 in air. Subconfluent cultures were infected with 5×10^6 *T. cruzi* trypomastigotes per 7.5 cm^2 flask. Free parasites were removed after 24 h by washing the cell monolayer. Cultures were maintained by adding fresh medium and after 5 days the culture supernatants containing trypomastigotes were collected [Schenkman et al., 1991]. To obtain amastigotes, the trypomastigotes released from the infected Vero cells were incubated in LIT medium for 48 h at 37°C, allowing extracellular transformation into amastigotes [Andrews et al., 1988]. *Crithidia fasciculata* strain ATCC11745 was obtained from the American Type Culture Collection and was grown in standard Brain Heart Infusion medium (DIFCO). Promastigotes of *Leishmania donovani* LD3 [Turco and Descoteaux, 1992], *L. major* 5-ASKH and *L. tropica* K-27 [Tolson et

al., 1994b] were obtained from Dr. S. Turco (University of Kentucky Medical Center, Lexington, KY, USA) and Dr. L. Schnur (Hadassah Medical School, Jerusalem, Israel), respectively, and were maintained in culture at 26°C in the same medium used to grow trypanosome PCF.

Transformation to procyclic culture forms. BSF of *T. congolense* IL3000 were harvested aseptically from rat blood, purified over a DEAE-cellulose column [Lanham and Godfrey, 1970] and washed twice by centrifugation (900 x g, 10 min, 4°C) with phosphate-buffered saline (PBS) (140mM NaCl, 2.7 mM KCl, 8.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4)/1% glucose. The parasites were adjusted to 5 x 10^6 ml^{-1} in procyclic culture medium and 60 ml placed into each of seven 250-ml tissue culture flasks. The flasks were incubated at 26°C and at each of seven intervals (0, 2, 4, 8, 24, 48 and 72 hours) parasites were harvested from one flask, washed once by centrifugation (900 x g, 10 min, 4°C) with PBS/1% glucose, resuspended in 10 ml of the same buffer and counted to determine parasite concentrations. Parasites were pelleted and cell lysates prepared for subsequent gel electrophoresis and immunoblotting experiments (see below) by lysis of 1 x 10^5 parasites in 15 µl Laemmli sample buffer [Laemmli, 1970].

Polyacrylamide gel electrophoresis. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [1970] using a minigel apparatus (Mini Protean II, Biorad, Richmond, CA, USA). Proteins were stacked using a 3% gel and were separated on a 10% gel at 200 V. Parasite lysates were prepared for SDS-PAGE by lysis of 3 x 10^6 parasites in 15 µl Laemmli sample buffer. Samples (15 µl) were applied to each gel. Molecules were stained with Coomassie blue R-250, silver [Merrill et al., 1984] or were detected by immunoblotting (see below) or autoradiography (also see below). For protein microsequencing, SDS-PAGE minigels were run under special conditions using recrystallized SDS as described by Matsudaira [1987]. Rainbow™ coloured protein molecular mass markers (Amersham, Oakville, Ontario, Canada) were run on each gel. For autoradiography of ^{125}I-labeled proteins, SDS-PAGE minigels were treated with 20% Trichloroacetic acid (TCA) for 45 min and 3% glycerol for 1 h prior to drying of the gel (model 483 slab dryer, Biorad, Richmond, CA, USA) and autoradiography.

Electrophoretic blotting. Electrophoretic transfer of proteins from SDS-PAGE minigels onto polyvinylidene difluoride membranes (Immobilon-P™, Millipore Corp., Bedford, MA, USA) and subsequent detection of antigens were performed by the procedure of

Towbin et al. [1979], extensively modified to reduce background binding of antibodies [Birk and Koepsell, 1987] and to allow blotted antigens to renature [Bestagno et al., 1987]. Bound antibody was detected using a 1:3000 dilution of horseradish peroxidase-labeled goat anti-mouse IgG/IgM antibody (Caltag, South San Francisco, CA, USA). Enhanced luminol chemiluminescence reagent (Renaissance™, DuPont NEN, Boston, MA, USA) was used as the substrate for horseradish peroxidase according to the instructions of the manufacturer. For detection of biotin-labeled proteins, a 1:3000 dilution of streptavidin alkaline phosphatase conjugate (Amersham, Oakville, Ontario, Canada) was used. The 5-Bromo-4-Chloro-3'-Indolyl Phosphate (BCIP) p-Toluidine salt and Nitro-Blue Tetrazolium (NBT) chloride substrate combination for alkaline phosphatase was used as previously described [Moe and Kirkeby, 1982; Doria et al., 1988]. Tissue culture supernatant containing monoclonal antibody (mAb) 6A9 (see later under monoclonal antibodies and antiserum) was used as first antibody at a 1:2 dilution. For protein microsequencing, protein bands were located by staining the membranes with Coomassie blue R-250 [Matsudaira, 1987].

Protein microsequencing. Coomassie blue-stained bands of purified membrane proteins (see below) from Immobilon-P™ blots were placed directly into a gas-phase sequencer (model 470A, Applied Biosystems, Foster City, CA, USA) and sequence analysis was performed in the University of Victoria Tripartite Microanalytical Center. Sequences obtained were searched against the SWISS-PROT protein database [Altschul et al., 1990].

Biotin labeling and avidin affinity chromatography. T.b. rhodesiense ViTat 1.1 PCF were surface labeled with NHS-SS-biotin (Pierce, Rockford, IL, USA) using a procedure modified from that published by Busch et al. [1989]. Parasites (2×10^{10}) were washed once by centrifugation (900 x g, 10 min, 4°C) with PBS/1% glucose and resuspended to 2×10^9 ml^{-1} in the same buffer containing 1.0 mg NHS-SS-biotin. After 10 min on ice with repeated microscopic monitoring of parasite viability, the cells were washed twice with ice-cold PBS/1% glucose/10mM lysine, and the pellet was resuspended in 10 ml lysis buffer (20mM Tris-HCl (pH 8.0)/1 mM Na_2-EDTA/150mM NaCl/0.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)/0.02% NaN_3/10mM lysine/100µl protease inhibitor cocktail (1.56 mg ml^{-1} each of *N*-[*N*-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine (E-64), leupeptin, antipain, 62.5 µg ml^{-1} chymostatin) and lysed using a nitrogen bomb at 900 psi for 10 min. The insoluble material was separated by centrifugation (105,000 x g, 30 min, 4°C) and the supernatant

was incubated overnight at 4°C with gentle agitation with 5 ml (packed volume) of avidin-agarose (Pierce, Rockford, IL, USA) that had been equilibrated with 5 x 10 bed volumes of modified lysis buffer containing 0.1% CHAPS and no lysine. Unbound proteins were removed by washing with 5 x 10 bed volumes of modified lysis buffer, and the bound proteins were eluted with 4 x 1 bed volume of 20 mM dithiothreitol in modified lysis buffer. The eluate was dialyzed against water, lyophilized and solubilized in 400 µl Laemmli sample buffer prior to SDS-PAGE.

Double-labeling experiments. *T.b. rhodesiense* ViTat 1.1 PCF were double-labeled with sulfo-NHS-biotin (Pierce, Rockford, IL, USA) [Hurley et al., 1985] and ^{125}I (DuPont NEN, Boston, MA, USA). Parasites (1×10^9) were washed once by centrifugation (900 x g, 10 min, 4°C) with PBS/1% glucose and resuspended to 2×10^7 ml^{-1} in the same buffer containing 0.5 mg sulfo-NHS-biotin. Parasites were labeled for 10 min on ice with repeated microscopic monitoring of parasite viability, the cells were washed twice by centrifugation (600 x g, 10 min, 4°C) with PBS/1% glucose/10 mM lysine, and the resulting pellet was resuspended in 500 µl PBS/1% glucose. Finally, 400 µCi of carrier-free $Na^{125}I$ was added to the trypanosome suspension in a vial coated with 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (Iodo-Gen™; Pierce, Rockford, IL, USA) [Howard et al., 1982]. The parasites were incubated 4 min at 25°C with gentle agitation every minute, followed by microscopic assessment of parasite viability and the addition of 10 µl of 0.3 mg ml^{-1} tyrosine in PBS/1% glucose. Following a 1 min incubation, 10 ml of PBS/1% glucose was added and the mixture was pelleted by centrifugation (1500 x g, 10 min, 4°C). The labeled parasites were washed once with PBS/1% glucose by centrifugation (1500 x g, 10 min, 4°C) and the resulting pellet resuspended in 300 µl of Laemmli sample buffer prior to SDS-PAGE, immunoblotting and autoradiography.

Preparation of trypanosome membranes. A *T.b. rhodesiense* ViTat 1.1 PCF plasma membrane-enriched fraction was prepared as described by Siddiqui et al. [1990] modified only to exclude the final free-flow electrophoresis step. Parasites (1×10^{11}) were washed twice by centrifugation (10,000 x g, 10 min, 4°C) with ice-cold buffer A (10mM Tes buffer (pH 7.4)/0.25 M sucrose/5 mM $MgSO_4$/50 mM KCl/1 mM Na_2-EDTA). Washed parasites were resuspended to 1×10^9 ml^{-1} in ice-cold buffer A containing 100 µl protease inhibitor cocktail (see above) and sonicated on ice (8 x 15 s bursts). The insoluble material was removed by centrifugation (twice at 450 x g, 10 min, 4°C, followed by twice at 4500 x g, 10 min, 4°C). In each case the pellets were discarded and the supernatant retained. The microsomal fraction was found in the pellet upon ultracentrifugation at

105,000 x g for 60 min at 4°C and was washed twice with buffer A. To purify the plasma membranes from the microsomal fraction the washed pellet was resuspended in 17% Percoll™ and centrifuged at 35,000 x g for 90 min at 4°C. Plasma membranes were recovered in the density range 1.04-1.1045 mg ml^{-1} and excess Percoll™ was removed by layering on a 50% sucrose cushion followed by centrifugation at 105,000 x g for 60 min at 4°C. The plasma membrane fraction was recovered at the interface between the Percoll™ and the sucrose cushion. After washing twice with buffer A (105,000 x g, 60 min, 4°C) the pellet containing the plasma membranes was resuspended in 2 ml of distilled water and dialyzed overnight against 12 litres of distilled water. The sample was concentrated with 20 volumes of acetone, chilled overnight at -70°C, washed twice with 20 volumes of ice-cold acetone, dispensed into six tubes and dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA). The contents of the tubes were resuspended in a total of 500 µl Laemmli sample buffer for gel separation and blotting onto Immobilon-P™ membranes in preparation for protein microsequencing.

Agarose gel electrophoresis. Agarose gels were prepared by dissolving molecular biology grade agarose LE (Promega, Madison, WI, USA) in 1 x Tris-acetate (TAE; 0.04 M Tris-acetate, 0.001 M EDTA) buffer. Unless otherwise indicated 1% agarose gels were used. Either a lambda DNA-*Hind*III digest (New England Biolabs, Beverly, MA, USA) or a 1 kb DNA ladder (Gibco BRL, Burlington, ON, Canada) was run on each gel to serve as size standards. DNA bands were stained with 1.27 µM ethidium bromide (Sigma, St. Louis, MO, USA), visualized by viewing on a Fotodyne™ transilluminator and photographed using a Fotodyne™ camera (Bio/Can scientific, Mississauga, ON, Canada).

Cloning and sequencing. The *E. coli* strains DH5α (Gibco BRL, Burlington, ON, Canada), XL-1 Blue (tetracycline^R; Stratagene, La Jolla, CA, USA) and BL21(DE3)pLysS (chloramphenicol^R; Novagen, Madison, WI, USA) were used in transformation experiments according to the method of Hanahan et al. [1983] and standard gene cloning techniques were applied as described by Sambrook et al. [1989]. Bacterial cultures were routinely grown in either Luria-Bertani (LB) medium (1% bacto-tryptone, 0.5% bacto-yeast extract (both from Difco Laboratories, Detroit, MI, USA), 0.17 M NaCl, pH 7.0) or 2 x YT medium (1.6% bacto-tryptone, 1% bacto-yeast extract, 86 mM NaCl, pH 7.0) [Sambrook et al., 1989]. Amplified PCR fragments (see below) were recovered from low-melting-temperature agarose using Wizard™ PCR Preps purification system (Promega, Madison, WI, USA) and cloned into either the

Bluescript™ SK⁺ vector (Stratagene, La Jolla, CA, USA) or the pGEM-T™ vector (Promega, Madison, WI, USA). Single-stranded DNA was prepared using a modification of the protocol described by Vieira and Messing [1987]. Briefly, clones to be sequenced were inoculated into 5 ml of 2 x YT medium with 1 x 10^8 M13KO7 helper phage (Gibco BRL, Burlington, ON, Canada), the culture was grown at 37°C for 2 hours, kanamycin was added to 70 μg ml^{-1} and the culture was incubated at 37°C for a further 16 to 24 hours. Bacteria were pelleted and the resulting supernatant was precipitated with 1.5 volumes of 20% polyethylene glycol (PEG, Av. Mol. Wt. 8000; Sigma Chemical Company, St. Louis, MO, USA)/2.5 M NaCl (0°C, 15 min). The PEG precipitate was resuspended in 0.3 M NaOAc pH 6.0/1 mM EDTA (0.08 volumes of the original culture volume), the mixture was extracted with one volume of phenol/chloroform and the aqueous phase precipitated with 95% ethanol. The resulting single stranded DNA pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA; 0.005 volumes of the original culture volume). Double-stranded DNA was prepared using Nucleobond™ AX cartridges (Macherey-Nagel, Düren, Germany). Sequencing of both single-stranded and double-stranded DNA was performed using Sequenase™ Version 2.0 DNA sequencing kits (United States Biochemical, Cleveland, OH, USA). Double-stranded DNA was also sequenced using Applied Biosystems 373-A automated sequencers. Both dye primer and dye terminator cycle sequencing were performed according to the manufacturers' instructions. In all cases both universal sequencing primers and internal primers designed from DNA sequences were used for sequencing. The 5' pGEX Sequencing Primer and the T7 Sequencing Primer (Pharmacia, Piscataway, NJ, USA) were used for DNA sequencing of the pGEX-2T and pET-3d overexpression constructs, respectively (see below).

RNA-PCR. T.b. rhodesiense ViTat 1.1 PCF poly(A)⁺ RNA was isolated from trypanosome lysates by the procedure described in Sambrook et al. [1989] using poly-U-Sepharose (Pharmacia, Piscataway, NJ, USA) [Cox and Smulian, 1983]. All primers were designed using OLIGO™ Version 4.0 software (National Biosciences, Inc., Plymouth, MN, USA). First-strand cDNA was synthesized using the 3' rapid amplification of cDNA ends (3' RACE) system [Frohman et al., 1988]. Poly(A)⁺ RNA was reverse transcribed in a 20 μl reaction mixture consisting of 5 mM $MgCl_2$, 1x PCR buffer (100 mM Tris-HCl, 500 mM KCl), 1 mM each dATP, dCTP, dGTP and dTTP (Pharmacia, Piscataway, NJ, USA), 50 units reverse transcriptase (New England Biolabs, Beverly, MA, USA), 2.5 μM oligo(dT) adaptor primer

[5'-GACTCGAGTCGACATCG$(T)_{17}$ -3'] and 1 μg poly(A)$^+$ RNA (42°C, 40 min; 99°C, 5 min; 4°C, 5 min). For subsequent amplifications each reaction mixture (100 μl total volume) consisted of 0.25 μM forward primer, 0.25 μM reverse primer, 2 mM $MgCl_2$, 1 x PCR buffer, 20 μl cDNA (see above) and 2.5 units *Taq* DNA polymerase (Pharmacia, Piscataway, NJ, USA). Specific forward [5'-GGGGTTTAAGCTTGTGTCCGGTGTGACTTACCTGAAGCGCGGTGC-3'] (introduced a *Hind*III site, indicated by the underlined sequence) and reverse [5'-GGGGTTTGAATTCACCGCGGGACTCCACCTTCTTGGCGAGCAC-3'] (introduced an *Eco*RI site, indicated by the underlined sequence) primers were designed from the extreme ends of the 34 amino acid NH_2-terminal amino acid sequence obtained by gas-phase sequencing of the 38 kDa band on Immobilon-P™ (see sequence later in the results section). The oligonucleotides were constructed using trypanosome codon usage [Parsons et al., 1991]. The following polymerase chain reaction (PCR [Saiki et al., 1988]) protocol was performed in a Thermolyne Temp·Tronic™ Thermal Cycler (Barnstead/Thermolyne, Dubuque, IA, USA): 55°C annealing, 70°C extension, 2 cycles; 62°C annealing, 70°C extension, 30 cycles. The upstream noncoding region and a partial coding region were amplified using a 5' mini-exon (forward) primer [5'-CAGTTTCTGTACTATATTG-3'] [Borst, 1986; Walder et al., 1986] and a specific 3' internal (reverse) primer [5'-CAGAGCGGTACCGAA-3']. The following PCR protocol was performed: 40°C annealing, 74°C extension, 4 cycles; 45°C annealing, 74°C extension, 35 cycles. The coding and downstream noncoding regions were amplified using a specific 5' internal (forward) primer [5'-CTTCGGTACCGCTCTG-3'] and a 3' adaptor (reverse) primer [5'-GACTCGAGTCGACATCG-3']. The following PCR protocol was performed: 50°C annealing, 74°C extension, 37 cycles. In all amplifications "hot start" PCR [Chou et al., 1992] was performed by adding 2.5 units of *Taq* polymerase (Pharmacia, Piscataway, NJ, USA) after the initial denaturation step.

Genomic PCR. T.b. rhodesiense ViTat 1.1 PCF genomic DNA was isolated from trypanosome lysates by the procedure described in Sambrook et al. [1989]. The nucleotide sequence of the 38 kDa molecule was used to design forward and reverse PCR primers to amplify the coding region of the putative GPD from trypanosome genomic DNA. Primer 1 (forward) [5'-GGAATTCCATATGGTCTCCGGTGTGACATATCTGAAGC-3'] was complementary to the 5' end of the coding region and introduced an *Nde*I site (underlined sequence) at the ATG start codon. Primer 2 (reverse)

[5'-CGGGATCCCTAAATCCCACACCGCCC-3'] was complementary to the region immediately downstream of the 3' end of the coding region and introduced a *Bam*HI site (underlined sequence) 37 bp downstream of the natural stop codon. The PCR amplification reaction mixture (100 µl total volume) consisted of 0.25 µM forward primer, 0.25 µM reverse primer, 1 x *Taq* polymerase buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 2 mM each dATP, dCTP, dGTP and dTTP (Pharmacia, Piscataway, NJ, USA), 500 ng genomic DNA and 2.5 units *Taq* DNA polymerase (Pharmacia, Piscataway, NJ, USA). The following PCR protocol was performed: 69°C annealing, 74°C extension, 30 cycles. As for the RNA-PCR "hot start" PCR was performed.

Database searches, deduced amino acid sequence characterization and sequence alignments. Database searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm [Altschul et al., 1990] and either the dbEST database of GenBank™, a non-redundant DNA database (includes GenBank, GenBank updates, EMBL and EMBL updates) or a non-redundant protein database (includes SWISS-PROT, PIR, GenPept and GenPept updates). The nucleotide sequence was translated and analyzed using either the SeqApp™ software[1] or the GeneWorks® software (IntelliGenetics, Mountain View, CA, USA). The molecular mass was calculated using the MacProMass™ v1.05 software (Beckman Research Institute, City of Hope, Duarte, CA, USA). The isoelectric point (pI) and net charge at physiological pH (7.4) of the translated product were calculated using the PC/Gene™ software (IntelliGenetics, Mountain View, CA, USA). Alignment of the amino acid sequences of GPD from various organisms was carried out using the Clustal V™ general purpose multiple alignment program [Higgins et al., 1992]. The percentage of positional identity between sequences was calculated from the number of identical residues between aligned sequences; insertions and deletions were not counted. In each case the percentage was calculated from the GPD amino acid sequence with the least number of residues. The Prosite™ protein motif database was used to access the GPD signature pattern (Prosite™ accession number: PS00957).

Overexpression studies. To express the 38 kDa molecule as a recombinant fusion protein, forward and reverse PCR primers were designed from the nucleotide sequence

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[1] Gilbert, D. G. 1992. SeqApp™. Published electronically on the Internet, available via anonymous ftp from ftp.bio.indiana.edu. IUBio archive of molecular and general biology software and data.

of the 38 kDa molecule and used to amplify the coding region of the putative GPD from *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA in preparation for cloning into the Glutathione-S-Transferase (GST) gene fusion vector pGEX-2T (Pharmacia, Piscataway, NJ, USA). Primer 1 (forward) [5'-CGGGATCCGTCTCCGGTGTGACATATC-3'] was complementary to the 5' end of the coding region and introduced a *Bam*HI site (underlined sequence) one codon downstream of the ATG start codon. Primer 2 (reverse) [5'-CCGGAATTCCTATTCACGTTTTAGAATTGTTGGATC-3'] was complementary to the 3' end of the noncoding region and introduced an *Eco*RI site 228 bp downstream of the natural stop codon. The PCR amplification reaction mixture was prepared as described in the methods for genomic PCR. Following PCR (65°C annealing, 74°C extension, 30 cycles) the amplification product was digested with *Bam*HI and *Eco*RI, ligated to a similarly digested pGEX-2T overexpression vector and transformed into *E. coli* DH5α. Prior to overexpression, the nucleotide sequence spanning the pGEX-2T-putative GPD ligation junction was subjected to double-stranded DNA sequencing to verify the putative GPD sequence was in the correct reading frame for expression. Overexpression and subsequent purification of the GST-putative GPD fusion protein was performed according to the manufacturer's instructions. Briefly, a 1 litre DH5α/pGEX-2T-putative GPD culture was grown to an OD_{600} of 1-2, isopropylthio-β-D-galactosidase (IPTG) was added to a final concentration of 0.1 mM and the culture was incubated at 37°C for an additional 2-6 hours. Following centrifugation (8000 rpm, 10 min, 4°C) the bacterial pellet was resuspended in 50 ml PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and the bacteria were lysed by sonication on ice (setting 5, 8 x 30 s bursts). Triton X-100 was added to a final concentration of 1% and the sonicate was incubated 30 min at 4°C. Following centrifugation (13,120 x g, 10 min, 4°C) the supernatant was incubated for 30 minutes at room temperature with 1.5 ml of a 50% slurry of glutathione-Sepharose 4B (Sigma, St. Louis, MO, USA). After loading into a column the slurry was washed with 10 bed volumes of PBS pH 7.3 and the fusion protein eluted with 3 bed volumes of glutathione-elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0). To cleave the putative GPD away from the GST fusion protein, 75 cleavage units of thrombin (Sigma, St. Louis, MO, USA) in 2.0 ml PBS pH 7.3 was added to the column after washing with PBS pH 7.3. Following an overnight incubation the purified putative GPD protein was eluted from the column. Control overexpression studies were performed using the pGEX-2T vector alone. Protein samples resulting from the overexpression studies were prepared for SDS-PAGE

and immunoblotting analyses by the addition of an equal volume of 2x Laemmli sample buffer.

To express the 38 kDa molecule as a recombinant non-fusion protein, the coding region of the putative GPD was amplified by PCR from *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA, and the product was cloned into the pET-3d overexpression vector (Novagen, Madison, WI, USA). Primer 1 (forward) [5'-CATGCCATGGTCTCCGGTGTGACATATCTGAAGC-3'] was complementary to the 5' end of the coding region and introduced an *Nco*I site (underlined sequence) at the ATG start codon. Primer 2 (reverse) [5'-CGGGATCCCTAAATCCCACACCGCCC-3'] was complementary to the 3' end of the noncoding region and introduced a *Bam*HI site (underlined sequence) 37 bp downstream of the natural stop codon. The PCR amplification reaction mixture was prepared as described in the methods for genomic PCR. Following PCR (65°C annealing, 74°C extension, 30 cycles) the amplification product was digested with *Nco*I and *Bam*HI, ligated to a similarly digested pET-3d overexpression vector and transformed into *E. coli* DH5α. To verify that the putative GPD sequence was in the correct reading frame for expression, the 5' end of the ligation junction of the putative GPD-pET-3d construct was subjected to double-stranded DNA sequencing. To achieve target gene expression the putative GPD-pET-3d construct was subsequently cloned into the *E. coli* expression strain BL21(DE3)pLysS. Overexpression was performed according to the manufacturer's instructions. Briefly, a 0.1 litre culture (BL21(DE3)pLysS/pET-3D-putative GPD) was grown to an OD_{600} of 1-2, IPTG was added to a final concentration of 0.4 mM and the culture was incubated at 37°C for an additional 3 hours. Following a 5 min incubation on ice the culture was centrifuged (5000 x g, 5 min, 4°C) and the resulting bacterial pellet resuspended in 25 ml 50 mM Tris-HCl, pH 8.0, 2 mM Na_2-EDTA. Following centrifugation (5000 x g, 5 min, 4°C) the supernatant was discarded and the bacterial pellet, containing the overexpressed putative GPD, was resuspended in 500 µl H_2O. Controls were performed using the pET-3d vector alone. One microlitre of each of the resuspended bacterial pellets resulting from the pET-3d-putative GPD and pET-3d constructs were added to 14 µl of Laemmli sample buffer in preparation for SDS-PAGE and immunoblotting analyses.

Partial purification of the recombinant non-fusion putative GPD protein.
BL21(DE3)pLysS/pET-3D-putative GPD clones were grown as described above. Following IPTG addition and subsequent growth for 3 hours at 37°C the cultures were centrifuged (5000 x g, 5 min, 4°C) and the resulting bacterial pellet resuspended in 10 ml

50 mM Tris-HCl pH 8.0, 2 mM Na_2-EDTA. Lysozyme was added to a concentration of 100 μg ml^{-1}, followed by the addition of Triton X-100 to a final concentration of 0.1%. The mixture was incubated at 30°C for 15 min, sonicated on ice (2 x 10 s bursts) and centrifuged at 12,000 x g for 15 min at 4°C. The supernatant, or soluble fraction, was prepared for SDS-PAGE and immunoblotting by the addition of an equal volume of 2x Laemmli sample buffer. The pellet containing the insoluble fraction was resuspended in 500 μl Laemmli sample buffer and 1 μl of this sample was added to 14 μl of Laemmli sample buffer in preparation for SDS-PAGE and immunoblotting analyses.

Glycerol 3-phosphate dehydrogenase activity.[2] The GST-putative GPD fusion protein and the partially purified recombinant non-fusion putative GPD protein were tested for glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) activity at room temperature in 50 mM HEPES buffer (pH 7.8) in a final volume of 1 ml. Activity was determined with a Pye-Unicam SP6-550 UV/VIS spectrophotometer using the extinction of NADH at 340 nm (ϵ=6.22 x 10^3 mM^{-1} cm^{-1}). The conditions of the assay were optimized to ensure zero-order kinetics. The assay medium contained 0.12 mM NADH and 0.4 mM dihydroxyacetone phosphate (omitted for the control assay). Control rates of enzyme activity were negligible. One unit of enzyme activity was defined as 1 μmole of substrate produced per minute under assay conditions.

For kinetic analyses of GPD, the partially purified recombinant non-fusion protein was used. Samples were diluted with assay buffer (50 mM HEPES, pH 7.8). Analyses were performed using a kinetic microplate reader at room temperature and at nine dihydroxyacetone phosphate concentrations ranging from 0.064 to 3.2 mM. NADH was kept constant at 0.12 mM. Rabbit muscle GPD (Sigma Chemical Company, type III, Mississauga, ON, Canada) was assayed concurrently.

Monoclonal antibodies and antiserum. BALB/c mice were immunized by intraperitoneal injections with 25 μg of the purified fusion protein as described elsewhere [Pearson et al., 1980]. At the time of euthanasia peripheral blood was collected from the mice to provide an antiserum against the fusion protein. Fusion of mouse spleen cells with X63-Ag8.6.5.3 BALB/c parental myeloma cells was performed as previously described [Pearson et al., 1980]. One-step selection and cloning of hybridomas was performed in

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[2] This work was performed in part by Glenn Cooper and Dr. Tom Mommsen, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

ClonaCell HY™ methylcellulose (Stem Cell Technologies Inc., Vancouver, BC, Canada). The ClonaCell HY™ method allows HAT selection and cloning in a single step and eliminates the overgrowth of slower growing desirable clones by faster growing, perhaps undesirable, hybridomas. Hybridoma tissue culture supernatants were screened first on the fusion protein and glutathione-S-transferase (Sigma Chemical Company, Mississauga, ON, Canada) by enzyme-linked immunosorbent assay (ELISA), then on human transferrin by ELISA to eliminate hybridomas secreting non-specific, "sticky" mAbs and finally on *T.b. rhodesiense* ViTat 1.1 PCF lysates after SDS-PAGE and immunoblotting [Beecroft et al., 1993]. Hybridomas secreting antibodies of interest were cloned once more by growth in ClonaCell HY™ to ensure monoclonality. The anti-*T. brucei* procyclin mAb TBRP1/247 [Richardson et al., 1986; Richardson et al., 1988] and the anti-lipophosphoglycan (LPG) mAb CA7AE [Tolson et al., 1989] were used as controls in immunofluorescence studies. The anti-human transferrin mAb TH1 (T. W. Pearson and R. P. Beecroft, unpublished) was used as a control in ELISA.

Enzyme-linked immunosorbent assay (ELISA). Indirect ELISA was performed using a variety of solid-phase adsorbed antigens. Eluate from the glutathione-Sepharose 4B column containing the fusion protein, glutathione-S-transferase and control antigen (human transferrin) were coated onto wells of ELISA plates by drying 100-µl volumes of 10 µg ml^{-1} onto the wells. All indirect ELISAs were performed using standard methods [Engvall and Perlmann, 1971]. The first antibody consisted of either a 1:2 dilution of hybridoma tissue culture supernatant, a 1:1000 dilution of anti-fusion protein antiserum (positive control), or a 1:2000 dilution of anti-human transferrin mAb TH1 (negative control). A 1:2000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG/IgM (Caltag, South San Francisco, CA, USA) was used as second antibody. The substrate used was p-nitrophenyl phosphate (Sigma Chemical Company, Mississauga, ON, Canada).

Immunofluorescence. Indirect immunofluorescence was performed on suspensions of live *T.b. brucei* 427 PCF as previously described [Pearson et al., 1981]. Immunofluorescence using acetone-permeabilized *T.b. brucei* 427 PCF was performed by air-drying parasites onto glass slides and fixing for 30 min in acetone which had been pre-chilled to -20°C, followed by processing as previously described [Pearson et al., 1981]. The first antibody consisted of either a 1:2 dilution of hybridoma tissue culture supernatant containing mAb 6A9, a 1:1000 dilution of ascites fluid containing the anti-*T. brucei* procyclin mAb (TBRP1/247, positive control) or a 1:2 dilution of hybridoma tissue

culture supernatant containing the anti-LPG mAb (CA7AE, negative control). A 1:40 dilution of a mixture of affinity purified goat anti-mouse IgG-fluorescein isothiocyanate (FITC) and goat anti-mouse IgM-FITC (Caltag, South San Francisco, CA, USA) was used as the second antibody. Coverslips were applied over mountant containing a fluorescence fading inhibitor (Slowfade™-Light Antifade Kit, Cat. No. S-7461, Molecular Probes, Inc., Eugene, OR, USA). Immunofluorescence was observed using a Zeiss standard binocular microscope fitted with an epifluorescence attachment and a Zeiss NeoFluor 63/1.25 oil immersion objective. Photographs were taken using a 35-mm photomicrographic camera.

Immunoelectron microscopy.[3] *T.b. brucei* 427 BSF or PCF were processed for Lowicryl K4M embedding at -20°C as previously described [Frevert et al., 1992]. Thin sections were cut with a RMC MT-7 ultramicrotome and sequentially labeled with mAb 6A9 hybridoma tissue culture supernatant (1:1 dilution) and a 1:30 dilution of protein A gold (10 nm, Amersham, Arlington Heights, IL). Photographs were taken with a Zeiss EM 910 electron microscope.

Glycosome isolation. T.b. rhodesiense ViTat 1.1 PCF glycosomes were prepared according to the method of Aman and Wang [1986]. *T.b. rhodesiense* ViTat 1.1 PCF were resuspended to 2 x 10^9 ml^{-1} in phosphate buffered saline/glucose (PSG) buffer (60 mM Na_2HPO_4, 3 mM NaH_2PO_4, 40 mM NaCl and 25 mM glucose) containing 2 μg ml^{-1} leupeptin. Following a 10 minute incubation at 37°C the mixture was centrifuged (1500 x g, 10 min, 4°C) and the resulting pellet was resuspended in 5 ml TEDS buffer (0.25 M sucrose, 25 mM Tris-Cl pH 7.5, 1 mM Na_2-EDTA, 1 mM dithiothreitol) containing 2 μg ml^{-1} leupeptin. After the addition of one cell weight of silicon carbide (Fisher Scientific Company, Fair Lawn, NJ, USA) the parasites were lysed by 30 strokes in a Wheaton™ homogenizer (model 358039, Fisher Scientific Company, Fair Lawn, NJ, USA) [Aman, and Wang, 1986]. Following centrifugation of the homogenate (1500 x g, 10 min, 4°C) the supernatant was retained and the pellet was washed two times with TEDS buffer (1500 x g, 10 min, 4°C). The supernatants were pooled and subjected to a final centrifugation (1500 x g, 10 min, 4°C) to sediment any remaining insoluble material. The resulting supernatant was centrifuged at 48,000 x g for 60 min at 4°C and the pelleted material was resuspended in 500 µl TEDS buffer and layered onto a discontinuous

[3] This work was performed by Dr. Ute Frevert, Department of Medical and Molecular Parasitology, New York University Medical Center, New York, NY, USA.

sucrose gradient (25, 35, 40, 50, 55 and 60% sucrose). After ultracentrifugation (230,000 x g, 70 min, 4°C) glycosomes were recovered at the interface between the 50% and 55% sucrose layers, diluted with TEDS buffer and pelleted (48,000 x g, 60 min, 4°C). Glycosomes were resuspended in 100 µl TES buffer (0.25 M sucrose, 25 mM Tris-Cl pH 7.5, 1 mM Na_2-EDTA) and diluted 1:1 with 2x Laemmli sample buffer for SDS-PAGE and immunoblot analysis.

Southern and Northern blot analyses. For Southern blot analysis, 8 µg of *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA were digested overnight at 37°C with 100 units of each of *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Nde*I, *Sau*3AI and *Xho*I (New England Biolabs, Beverly, MA, USA), followed by a further 4 hour incubation at 37°C with an additional 100 units of enzyme to ensure complete digestion. The digests were electrophoresed for 11 hours at 15 V on a 0.6% agarose gel, using as size standards a lambda DNA-*Hind*III digest (New England Biolabs, Beverly, MA, USA). For Northern blot analysis, 0.9 µg of *T.b. rhodesiense* ViTat 1.1 PCF poly(A)$^+$ RNA was electrophoresed for 15 hours at 20 V on a 1% formaldehyde gel using established protocols [Sambrook et al., 1989]. A 0.24-9.5 Kb RNA ladder (Gibco BRL, Burlington, ON, Canada) was run simultaneously in order to accurately establish transcript size. The genomic DNA restriction digests and the poly(A)$^+$ RNA were transferred via vacuum blotting (model 785, BioRad, Hercules, CA, USA) to Zeta-Probe® membrane (BioRad, Hercules, CA, USA) according to the manufacturer's instructions with slight modifications. Briefly, the agarose gel for Southern blot analysis was treated with 0.25 N HCl (1 x 15 min), 1.5 M NaCl/0.5 M NaOH (2 x 15 min) and 1.5 M NaCl/0.5 M Tris-HCl pH 7.5 (1 x 30 min) prior to transfer in 0.5 M NaOH/0.6 M NaCl. Twenty five nanograms of the genomic PCR fragment corresponding to the entire GPD coding region (see genomic PCR methods) was labeled with [α-^{32}P]dATP (specific activity 3000 Ci/mmol) using the Multiprime DNA labeling system (Amersham, Oakville, ON, Canada) according to the manufacturer's instructions. The Southern and Northern blots were prehybridized 5 minutes at 50°C in 0.25 M Na_2HPO_4, 7% SDS and hybridized overnight at 50°C with mild agitation in the same buffer with the labeled homologous probe (1.3×10^{-6} c.p.m. ml^{-1}; specific radioactivity 1.8×10^9 c.p.m. µg^{-1}). The membranes were washed 2 times for 45 minutes each at 50°C with 200 ml of 20 mM Na_2HPO_4 pH 7.2, 5% SDS, followed by 2 washes for 20 minutes each at 50°C with 200 ml of 20 mM Na_2HPO_4 pH 7.2, 1% SDS. The membranes were then air-dried and autoradiographed.

Bacteriophage P1 library screening. A bacteriophage P1 library high density filter (SM7 No 8) containing DNA from *L. donovani* strain 2903 and *T. brucei* strain 927 [Turner et al., 1990] was kindly provided by Drs. Sara Melville and Vanessa Leech (Cambridge University, Department of Pathology, Laboratory for Parasite Genome Analysis, Cambridge, England). The library was constructed in the p*AD10*SacBII cloning vector as previously described [Pierce et al., 1992]. Briefly, *L. donovani* and *T. brucei* genomic DNA was partially digested with *Sau*3AI to generate inserts of approximately 60-70 kb which were subsequently cloned into the *Bam*HI site of the vector. The library was grown in 96-well microtitre plates and the colonies in each well were subsequently transferred to a high density filter (Hybond N, Amersham, England). The high density filter was probed using the α-^{32}P-labeled GPD genomic PCR fragment probe generated for the Southern and Northern blot analyses (described above). Prehybridization, hybridization, filter washing and autoradiography were performed as described for the Southern and Northern blot analyses.

Processing of bacteriophage P1 clones.[4] P1 clones demonstrating reactivity with the homologous probe were selected and DNA was subsequently isolated from these clones using conditions suggested by the Laboratory for Parasite Genome Analysis [Birnboim and Doly, 1979]. The DNA was denatured (95°C, 3 min) and subsequently dotted onto Zeta-Probe® membrane (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Dot blots were rescreened using the homologous GPD probe under conditions exactly as outlined for the Southern and Northern blot analyses to verify positivity. In order to decrease the insert size of the P1 clone in preparation for obtaining flanking regions of the GPD gene, DNA was isolated from one positive P1 clone from the second screen, digested with *Eco*RI (New England Biolabs, Beverly, MA, USA) and separated on a 0.6% agarose (Promega, Madison, WI, USA) gel. The DNA was transferred to Zeta-Probe® membrane (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, probed with the homologous GPD gene as described for the Southern and Northern blot analyses and one hybridizing DNA fragment was selected. Although beyond the scope of this thesis, future work will use these genomic flanking sequences for knockout mutagenesis of the GPD gene.

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[4] This work was performed in part by Michael Bridge, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

RESULTS

Surface labeling analyses. To identify molecules present on the parasite cell membrane, *T.b. rhodesiense* ViTat 1.1 PCF were subjected to a series of mild surface labeling experiments using a variety of reagents. Parasites were first labeled with the primary amine-reactive biotinylation reagent NHS-SS-biotin, followed by passage of the parasite lysate over an immobilized avidin support and removal of unlabeled proteins by excess washing. Biotinylated cell surface proteins retained on the column were subsequently cleaved from the biotin label/immobilized avidin by reduction of the disulfide bond located in the spacer arm of the biotinylation reagent, thus yielding a preparation of cell surface proteins. Figure 3, panel A shows the protein profile obtained upon SDS-PAGE and silver staining of this preparation. In a separate experiment parasites were double labeled with ^{125}I and the primary amine-specific biotinylation reagent sulfo-NHS-biotin, and cell surface proteins incorporating both labels were detected by a combination of SDS-PAGE and electrophoretic blotting with subsequent autoradiography and biotin label detection. The ^{125}I- and sulfo-NHS-biotin-labeled protein profiles obtained are shown in Figure 3 panels B and C, respectively. The protein profiles from the cell surface labeling experiments were compared and proteins identified by labeling in all three experiments were selected for further analysis (protein bands 1 to 5, indicated by arrowheads in Figure 3).

Membrane protein purification and NH_2-terminal amino acid sequencing. A plasma membrane-enriched membrane preparation was isolated from *T.b. rhodesiense* ViTat 1.1 PCF and subjected to SDS-PAGE, electrophoretic transfer to Immobilon-P™ and Coomassie blue staining (Figure 3, panel D). The bands 1 to 5 identified as prospective cell surface proteins by the above experiments were selected for gas-phase NH_2-terminal amino acid microsequencing. Protein bands 2 and 5 were found to have blocked NH_2-termini and analysis of these proteins was discontinued at this point. NH_2-terminal sequences were successfully obtained for protein bands 1 (~50 kDa), 3 (~40 kDa) and 4 (~38 kDa), and these sequences are listed in Table 4. Comparison of these sequences with the SWISS-PROT protein database identified protein band 1 as the α-subunit of tubulin (accession number: P10489), whereas protein bands 3 and 4 at the time revealed no significant similarities with other known proteins in the database. A recent search performed on the sequence obtained for band 3 has identified this protein as glyceraldehyde 3-phosphate dehydrogenase (accession number: P22512). The NH_2-terminal sequence of protein band 4 was especially interesting as this sequence was of a

Figure 3 SDS-PAGE analyses of *T.b. rhodesiense* ViTat 1.1 PCF surface labeled proteins and plasma membrane-enriched preparation. Protein molecular mass markers (in kilodaltons) are indicated on the left of each panel. **A.** Silver stained SDS-PAGE gel profile of immobilized avidin eluate containing NHS-SS-biotin-labeled proteins. **B.** Autoradiograph of ^{125}I-labeled proteins. **C.** Biotin detection blot of sulfo-NHS-biotin-labeled proteins. **D.** Coomassie blue stained SDS-PAGE gel profile of plasma membrane-enriched preparation. The protein bands that were selected for NH_2-terminal amino acid sequencing are indicated by arrowheads and numerically labeled.

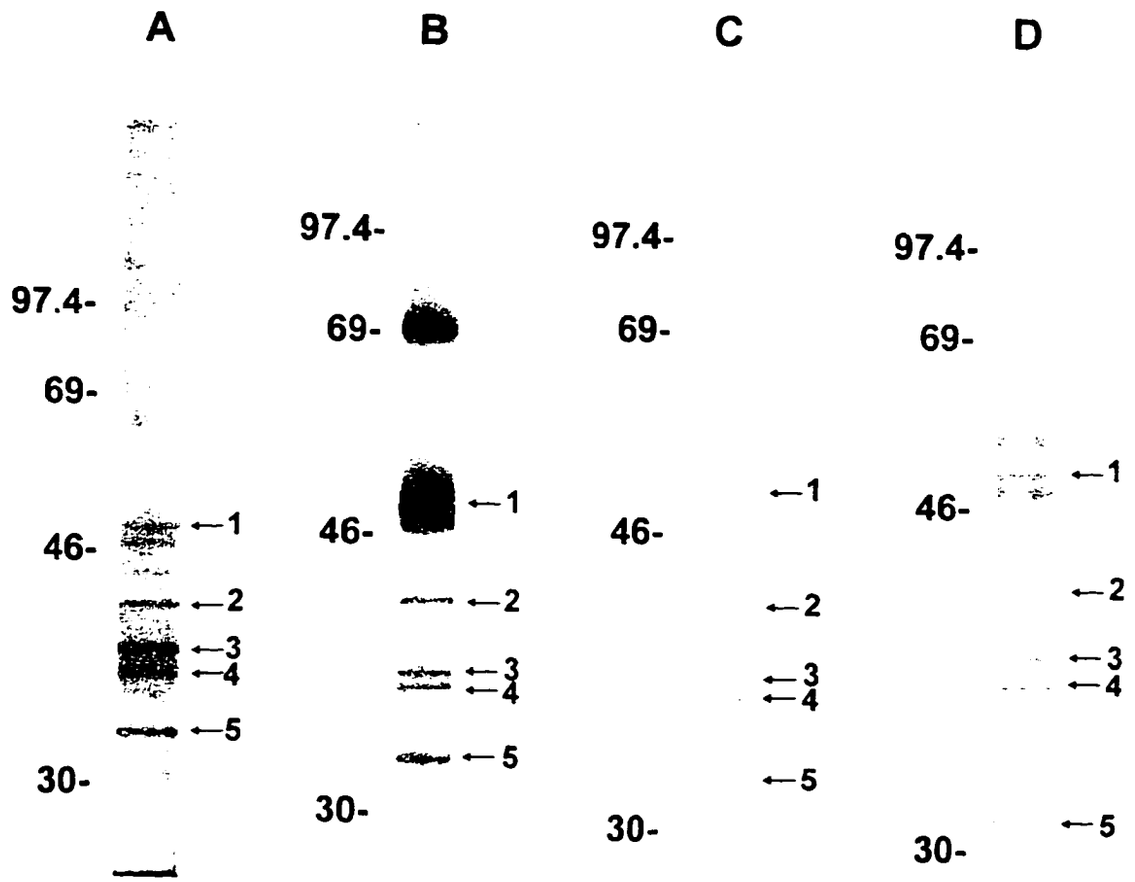

A
B
C
D
97.4-
69-
46-
30-
←1
←2
←3
←4
←5

Table 4 NH_2-terminal amino acid sequences of protein bands from a Coomassie blue stained Immobilon-P™ blot of *T.b. rhodesiense* ViTat 1.1 PCF plasma membrane-enriched preparation.

CYCLE NUMBER	AMINO ACID RESIDUE		
	BAND 1 (~50 kDa)	BAND 3 (~40 kDa)	BAND 4 (~38 kDa)
1	?	I	V
2	R	R	S
3	E	G	G
4	A	V	V
5	I	G	T
6	?	Y	Y
7	I	N	L
8	H	G	K
9	I	R	R
10	G	G	G
11	Q	R	A
12	A	V	V
13	G	G	F
14	?	R	G
15	Q	A	S
16		V	G
17			A
18			F
19			G
20			T
21			A
22			L
23			A
24			R*
25			V
26			L
27			A
28			K
29			K
30			V*
31			E
32			S
33			R*
34			G*

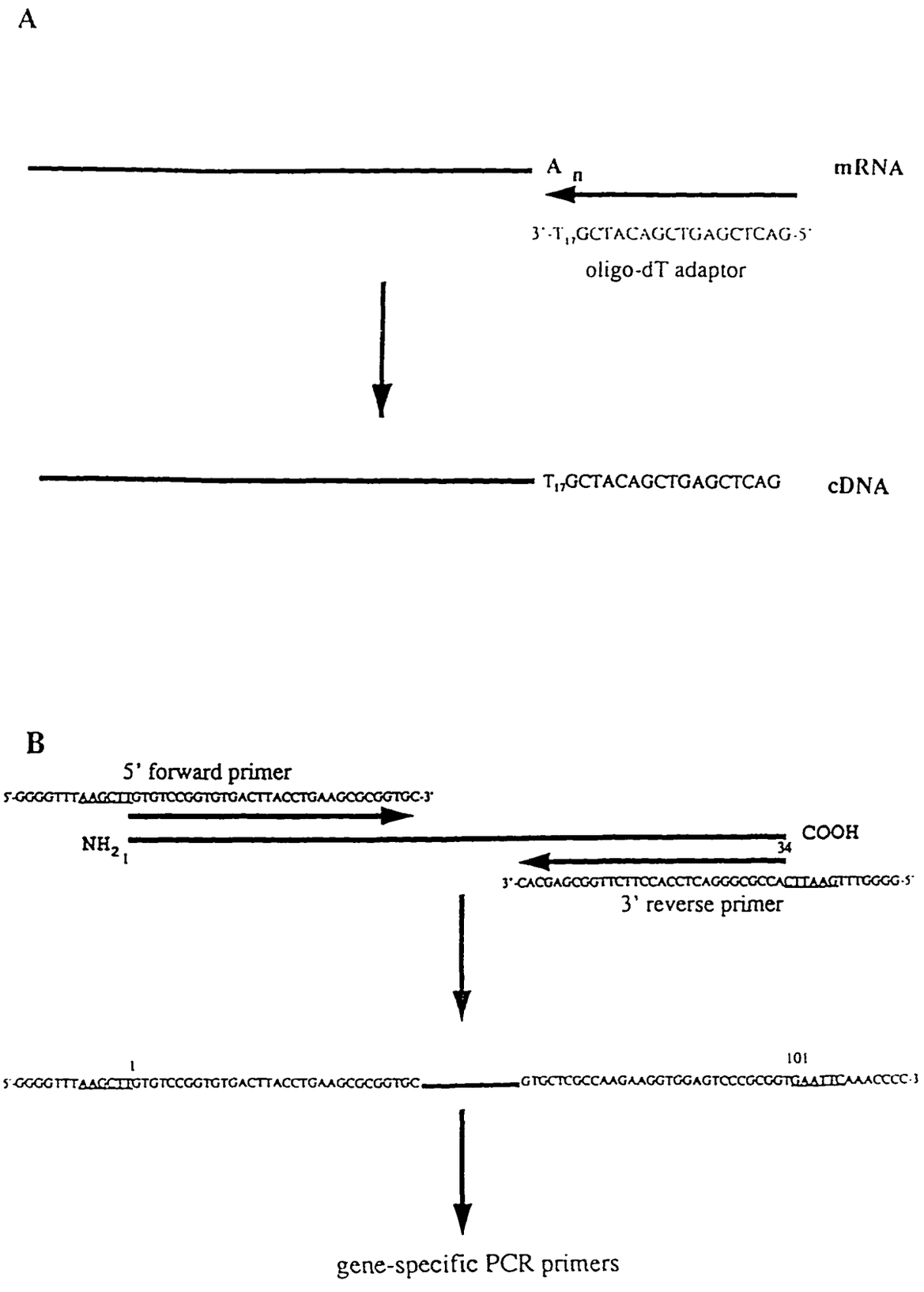
? identity of residue could not be determined.
* identity of residue was found to be incorrect (based upon subsequent determination of the complete DNA sequence encoding this protein).

favorable length for designing of primers for PCR and showed minimal ambiguity in the identity of the amino acid residues. This 38 kDa trypanosome protein was selected for further study.

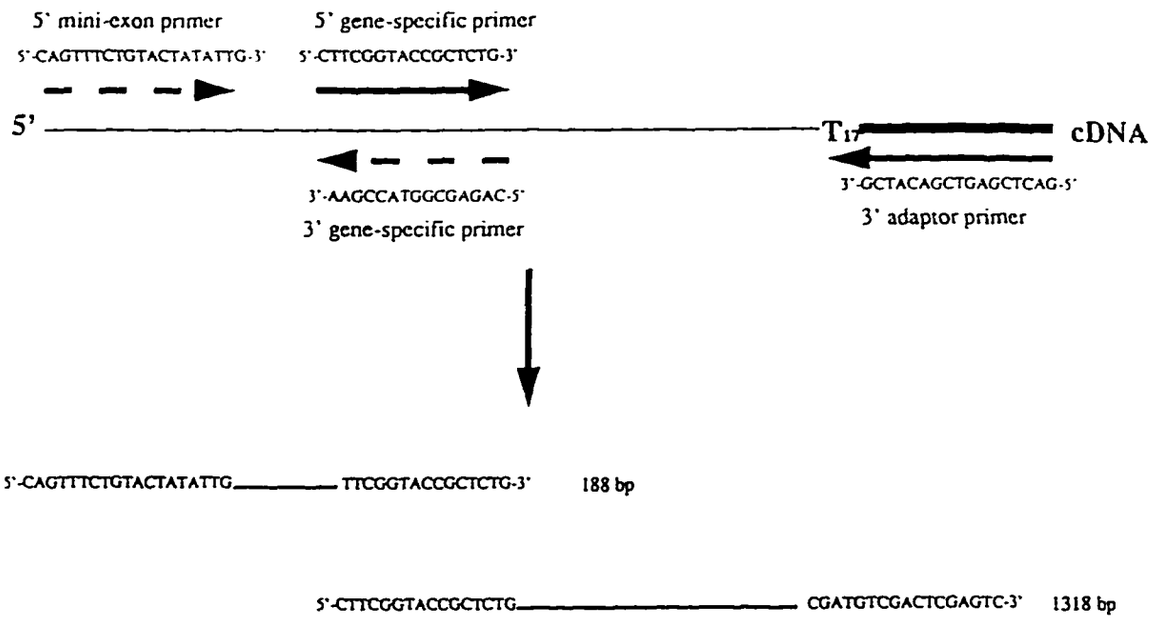
Surface disposition and NH_2-terminal amino acid sequence verification. Prior to proceeding with investigations on the 38 kDa protein isolated from the plasma membrane preparation, a confirmation of its identity as the same 38 kDa protein identified through the surface labeling studies was performed. This verification was achieved by cell surface NHS-SS-biotin labeling and subsequent purification of the biotin-labeled proteins using avidin-agarose affinity chromatography as outlined above, followed by separation of the resulting protein preparation by SDS-PAGE, transfer to Immobilon-P™ and Coomassie-blue staining. The 38 kDa protein present in this preparation was excised and subjected to NH_2-terminal amino acid microsequencing. A 20 residue sequence was obtained that exactly matched the sequence shown in Table 4 for the 38 kDa protein isolated from the plasma membrane preparation.

Nucleotide sequence encoding the 38 kDa protein. The entire nucleotide sequence encoding the trypanosome 38 kDa protein, as well as upstream and downstream flanking sequences, were obtained through a series of RNA- and genomic PCR amplifications. The PCR amplification strategy is illustrated in Figure 4, panels A-D. The initial round of PCR amplifications utilized RNA-PCR and amplified a 101 bp cDNA fragment corresponding to the NH_2-terminal amino acid sequence using PCR primers designed from the termini of this sequence (Figure 5, panel A, lane 1). This PCR fragment was cloned into the Bluescript™ SK^+ vector (Figure 5, panel A, lane 2). Sequencing of this PCR product revealed a 37 bp segment located internal to the two primer sequences and represents the true gene sequence free of primer-induced errors. This sequence was used to design specific reverse and forward PCR primers which, when used in conjunction with the mini-exon (forward) primer and the adaptor (reverse) primer, respectively, allowed the amplification by RNA-PCR of the entire cDNA sequence. The upstream noncoding sequence and the first 69 bp of the coding sequence were contained within a 188 bp PCR product (Figure 5, panel B, lane 1), while a larger 1318 bp PCR product contained the remaining coding and downstream noncoding sequences (Figure 5, panel C, lane 1). Each of these PCR products was cloned into the pGEM-T™ vector (Figure 5, panel B, lane 2 and Figure 5, panel C, lane 2, respectively) and the nucleotide sequence determined. This cDNA sequence was used to design PCR primers to amplify the entire coding sequence from *T.b. rhodesiense* genomic DNA (Figure 5, panel D, lane 1), which

Figure 4 PCR amplification strategy for isolation of both the complementary and genomic DNA fragments encoding the *T.b. rhodesiense* 38 kDa protein. **A.** 3'-RACE (rapid amplification of cDNA ends) cDNA synthesis utilizing the oligo-dT adaptor primer. **B.** PCR primer design for amplification of the cDNA fragment encoding the 34 residue NH_2-terminal amino acid sequence and subsequent design of gene-specific PCR primers (101 bp PCR fragment, sense strand only shown). **C.** PCR primer design for amplification of the cDNA fragments corresponding to the upstream non-coding and partial coding sequence (188 bp PCR fragment, sense strand only shown, primers used indicated by broken arrowheads) and the remaining coding and downstream non-coding sequence (1318 bp PCR fragment, sense strand only shown, primers used indicated by solid arrowheads). **D.** PCR primer design for amplification of the genomic DNA fragment corresponding to the entire coding and partial downstream non-coding sequence (sense strand only is shown). The open reading frame encoding the 38 kDa trypanosome protein and the natural stop codon (*) are indicated.


A
A n
mRNA
3'-T17GCTACAGCTGAGCTCAG-5'
oligo-dT adaptor
T17GCTACAGCTGAGCTCAG
cDNA
B
5' forward primer
5'-GGGGTTTAAGCTTGTGTCCGGTGTGACTTACCTGAAGCGCGGTGC-3'
NH2
1
COOH
34
3'-CACGAGCCGGTTCTTCCACCTCAGGGCGCCACTTAAGTTTGGGG-5'
3' reverse primer
1
101
5'-GGGGTTTAAGCTTGTGTCCGGTGTGACTTACCTGAAGCGCGGTGC
GTGCTCGCCAAGAAGGTGGAGTCCCGCGGTGAATTCAAACCCC-3
gene-specific PCR primers

C


5' mini-exon primer
5'-CAGTTTCTGTACTATATTG-3'
5' gene-specific primer
5'-CTTCGGTACCGCTCTG-3'
5'
T17
cDNA
3'-AAGCCATGGCGAGAC-5'
3' gene-specific primer
3'-GCTACAGCTGAGCTCAG-5'
3' adaptor primer
5'-CAGTTTCTGTACTATATTG
TTCGGTACCGCTCTG-3'
188 bp
5'-CTTCGGTACCGCTCTG
CGATGTCGACTCGAGTC-3'
1318 bp

D

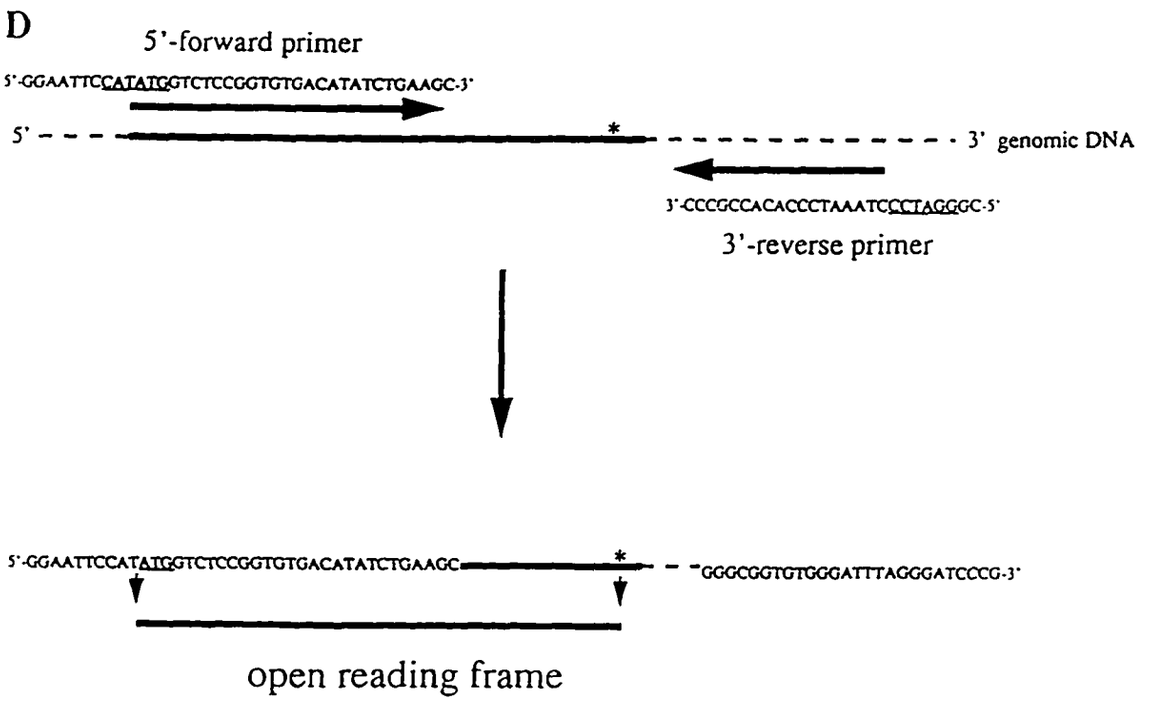
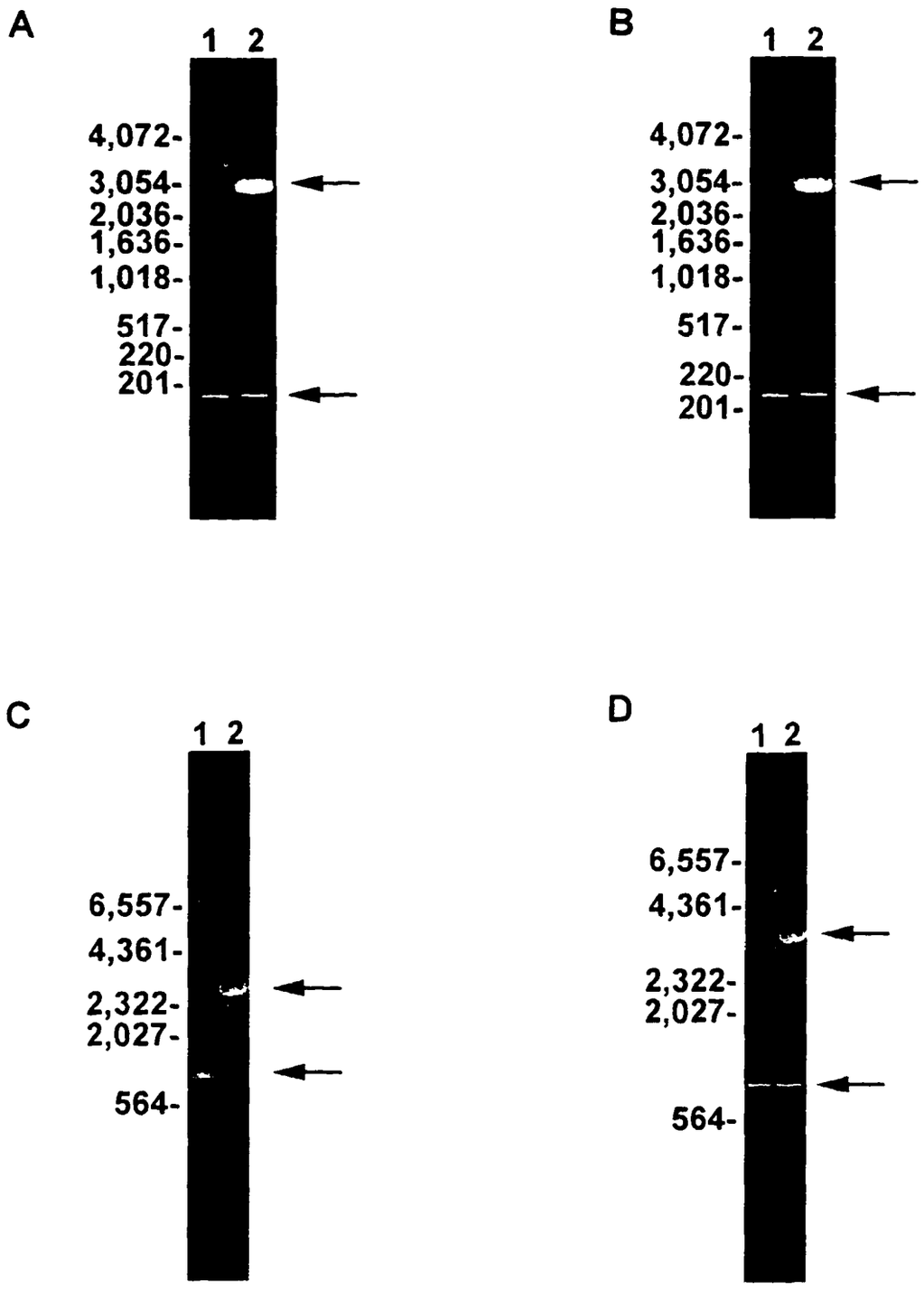

5'-forward primer
5'-GGAATTCCATATGGTCTCCGGTGTGACATATCTGAAGC-3'
5'
*
3' genomic DNA
3'-CCCGCCACACCCTAAATCCCTAGGGC-5'
3'-reverse primer
5'-GGAATTCCATATGGTCTCCGGTGTGACATATCTGAAGC
*
GGGCGGTGTGGGATTTAGGGATCCCG-3'
open reading frame

Figure 5 Agarose gel analyses of the amplified PCR fragments corresponding to the DNA encoding the 38 kDa protein (lower arrows) and the PCR fragments cloned into sequencing vectors (upper arrows). For visualization purposes the PCR fragments were excised from their respective cloning vectors by digesting with the indicated restriction endonucleases. Molecular size standards (in base pairs) are indicated on the left of each panel. **A.** Lane 1, the 101 bp PCR fragment corresponding to the NH_2-terminal amino acid sequence. Lane 2, the 101 bp PCR fragment cloned into the Bluescript™ SK+ vector (excised with *Hind*III and *Eco*RI). **B.** Lane 1, the 188 bp PCR fragment corresponding to the upstream non-coding and partial coding sequence. Lane 2, the 188 bp PCR fragment cloned into the pGEM-T™ vector (excised with *Nco*I and *Nde*I). **C.** Lane 1, the 1318 bp PCR fragment corresponding to the remaining coding and downstream non-coding sequence. Lane 2, the 1318 bp PCR fragment cloned into the pGEM-T™ vector (excised with *Nco*I and *Nde*I). **D.** Lane 1, the genomic PCR fragment corresponding to the entire coding and partial downstream non-coding sequence. Lane 2, the genomic PCR fragment cloned into the pGEM-T™ vector (excised with *Nco*I and *Nde*I).


A
1 2
4,072-
3,054-
2,036-
1,636-
1,018-
517-
220-
201-
B
1 2
4,072-
3,054-
2,036-
1,636-
1,018-
517-
220-
201-
C
1 2
6,557-
4,361-
2,322-
2,027-
564-
D
1 2
6,557-
4,361-
2,322-
2,027-
564-

was subsequently cloned into the pGEM-T™ vector (Figure 5, panel D, lane 2) and sequenced. This latter PCR amplification, cloning and sequencing step was performed in order to confirm the DNA sequence encoding the 38 kDa protein. Figure 6 shows the nucleotide sequence determined from sequencing both the cDNA and genomic PCR fragments and the corresponding translated amino acid sequence encoded by the 1062 bp open reading frame. The 353 residue translated product (not counting the initiator methionine) has a calculated molecular mass of 37,745 Da, a pI of 9.0 and a net charge of +9 at physiological pH. The translated amino acid sequence matched the NH_2-terminal amino acid sequence obtained by protein microsequencing in all but four residues (Table 4; underlined sequence in Figure 6).

Sequence alignment analyses. Searching of the DNA database with the 1062 bp open reading frame using the BLAST algorithm revealed no significant similarities to other known DNA sequences. However, searching of the protein database with the translated amino acid sequence using the BLAST algorithm identified NAD^+-dependent glycerol 3-phosphate dehydrogenase (GPD) from a variety of sources as the optimal scoring protein. Figure 7 illustrates the alignment of the putative trypanosome GPD with GPD from other sources and Table 5 tabulates the percentages of amino acid identity between the various GPD. The trypanosome sequence shares the highest identity with GPD from *E. coli* (32.2%) and only 24.6% with yeast, 22.2% with fruit fly, 22.7% with rabbit and 23.5% with human. The percentages are significantly below comparisons of yeast, fruit fly, rabbit and human GPD sequences (46.0%-92.0%) but well within the range exhibited by comparisons between *E. coli* and yeast, fruit fly, rabbit and human GPD sequences (24.8%, 22.4%, 24.8% and 24.2%, respectively). It is interesting that comparisons between the GPD molecule from two trypanosome spp. *T.b. rhodesiense* (this study) and *T.b. brucei* (Kohl et al., 1996), which represent human and cattle pathogens, respectively, show only 4 differences in the amino acid sequence (Asp169, Lys277, Val310, His311 vs Asn169, Gln277, Ala310, Asp311 for the *T.b. rhodesiense* and *T.b. brucei* sequences, respectively). Comparison of the *T.b. rhodesiense* GPD sequence reported in this paper with the *T.b. rhodesiense* GPD cDNA fragment contained within the dbEST database of GenBank™ showed 12 base pair differences over the 344 base pair cDNA fragment [El-Sayed et al., 1995]. These discrepancies in the expressed sequence tag may be due to inaccuracies resulting from single pass sequencing.

The amino acid residues of presumed importance in NAD^+-coenzyme and substrate binding predicted through secondary structure analysis performed on rabbit

Figure 6 Nucleotide sequence of the encoding DNA and deduced amino acid sequence of the 38 kDa trypanosome protein. The amino acids corresponding to the NH_2-terminus of the purified 38 kDa membrane protein determined by gas-phase microsequencing are underlined.

* indicates the termination codon.

```
ATGGTCTCCG GTGTGACATA TCTGAAGCGC GGTGCGGTGT TCGGCAGTGG CGCCTTCGGT   60
M  V  S  G  V  T  Y  L  K  R  G  A  V  F  G  S  G  A  F  G         20

ACCGCTCTGG CGTGCGTCCT TGCAAAGAAG TGTGAGTCTG TGTCCGTATG GCATATGAAC  120
T  A  L  A  C  V  L  A  K  K  C  E  S  V  S  V  W  H  M  N         40

GCCAATGAGG CCCGTGTTGT GAACCAGAAG CACGAAAACG TATATTTCCT TCCCGGTGCA  180
A  N  E  A  R  V  V  N  Q  K  H  E  N  V  Y  F  L  P  G  A         60

CCGCTTCCAG CTAACCTTAC CTTCACGGCC GATGCTGAGG AGTGCGCGAA AGGTGCGGAG  240
P  L  P  A  N  L  T  F  T  A  D  A  E  E  C  A  K  G  A  E         80

ATTGTCCTCT TCGTCATCCC GACGCAGTTC CTCCGCGGGT TCCTCCAGAA GAACAGCCAC  300
I  V  L  F  V  I  P  T  Q  F  L  R  G  F  L  Q  K  N  S  H        100

ATCCTCCGCA ACCACGTTGT TTCCCGCAAC GTGCCCGTGG TGATGTGCAG CAAGGGCATC  360
I  L  R  N  H  V  V  S  R  N  V  P  V  V  M  C  S  K  G  I        120

GAGCGCTCCT CCCTCCTCTT CCCCGCACAG ATCTTGGAGG AGTTCCTGCC CAACTATCCA  420
E  R  S  S  L  L  F  P  A  Q  I  L  E  E  F  L  P  N  Y  P        140

ATCGGTGTTA TTGCTGGCCC TTCATTCGCC ATTGAAGTTG CTAAGGGAAT GCTGACAAAT  480
I  G  V  I  A  G  P  S  F  A  I  E  V  A  K  G  M  L  T  N        160

GTTTGCACTG CCGCGGCCGA CATCGACATG GCACGAAAGA TTCAGAGAAT CATGACCACA  540
V  C  T  A  A  A  D  I  D  M  A  R  K  I  Q  R  I  M  T  T        180

TCCGACGGCT CCTTCCGTTG CTGGGCAACC ACCGATGTTA TCGGATGCGA GATCGCAAGC  600
S  D  G  S  F  R  C  W  A  T  T  D  V  I  G  C  E  I  A  S        200

GCCATGAAGA ATGTGCTTGC CATTGCCTCA GGTGCACTAA AGGGACTTGG AACTGAGAAC  660
A  M  K  N  V  L  A  I  A  S  G  A  L  K  G  L  G  T  E  N        220

AATGCCCGCG CTGCCCTCAT CTCTCGTGGT CTTCTCGAAA TCCGTGACCT GACCTTGGCA  720
N  A  R  A  A  L  I  S  R  G  L  L  E  I  R  D  L  T  L  A        240

CTTGGCGGCA CGGGTGAGGC TGTCTTCGGC CTTCCCGGCC TCGGTGACTT ACTGCTCACC  780
L  G  G  T  G  E  A  V  F  G  L  P  G  L  G  D  L  L  L  T        260

TGCTCTTCAG AACTCTCACG TAACTTTACG GTGGGCATGA AGCTCGGCAA AGGCATTTCC  840
C  S  S  E  L  S  R  N  F  T  V  G  M  K  L  G  K  G  I  S        280

CTCGAAGAGA TCAAGCGCAC CAGCAAGGCC GTTGCTGAGG GTGTTGCAAC CGCTGAGCCG  900
L  E  E  I  K  R  T  K  K  A  V  A  E  G  V  A  T  A  E  P        300

CTGGAGCGAC TCGCCAAGAA GCACAACGTG CACCTCCCCA TATGCCATGA GGTCTACAAT  960
L  E  R  L  A  K  K  H  N  V  H  L  P  I  C  H  E  V  Y  N        320

GTGTTGTATG CCAACGGTTG TGCCAAACGC TCGTTCAAGA AGCTCAACTC CTGCAAGTTG 1020
V  L  Y  A  N  G  C  A  K  R  S  F  K  K  L  N  S  C  K  L        340

GCTGATGAGG GGCTCCCAGC GCTCCCGCGC ACTTCCAAAA TGTAA                  1065
A  D  E  G  L  P  A  L  P  R  T  S  K  M  *                        354
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Figure 7 The deduced amino acid sequence of the 38 kDa *T.b. rhodesiense* (protozoan) protein and comparison with NAD^+-dependent GPD from *Escherichia coli* (bacterium) [Sofia et al., 1994], *Saccharomyces cerevisiae* (yeast) [Larsson et al., 1993], *Drosophila virilis* (fruit fly) [Arai et al., 1988], *Oryctolagus cuniculus* (rabbit) [Otto et al., 1980] and *Homo sapiens* (human)[5]. Sequence identities are indicated by boxed regions and re-alignments to maximize sequence identity by dashes. The amino acids determined by direct microsequencing of the NH_2-terminus of the purified 38 kDa membrane protein are italicized and also indicated by the large bracket above the top line in the Figure. The 22 amino acid Prosite™ GPD signature pattern [G-A-(LIVM)-K-(DN)-(LIVM)-(LIVM)-A-x-(GA)-x-G-(LIVMF)-x-(DE)-G-(LIVM)-x-(LIVMFYW)-G-x-N] is underlined. Amino acids of presumed importance in NAD^+-coenzyme and substrate binding are indicated above the amino acid symbols. See text for further explanation.

[5] Menaya, J., Gonzalez-Manchon, C., Ayuso, M. S. and Parrilla, R. 1994. Unpublished results, BLAST accession number: L34041.

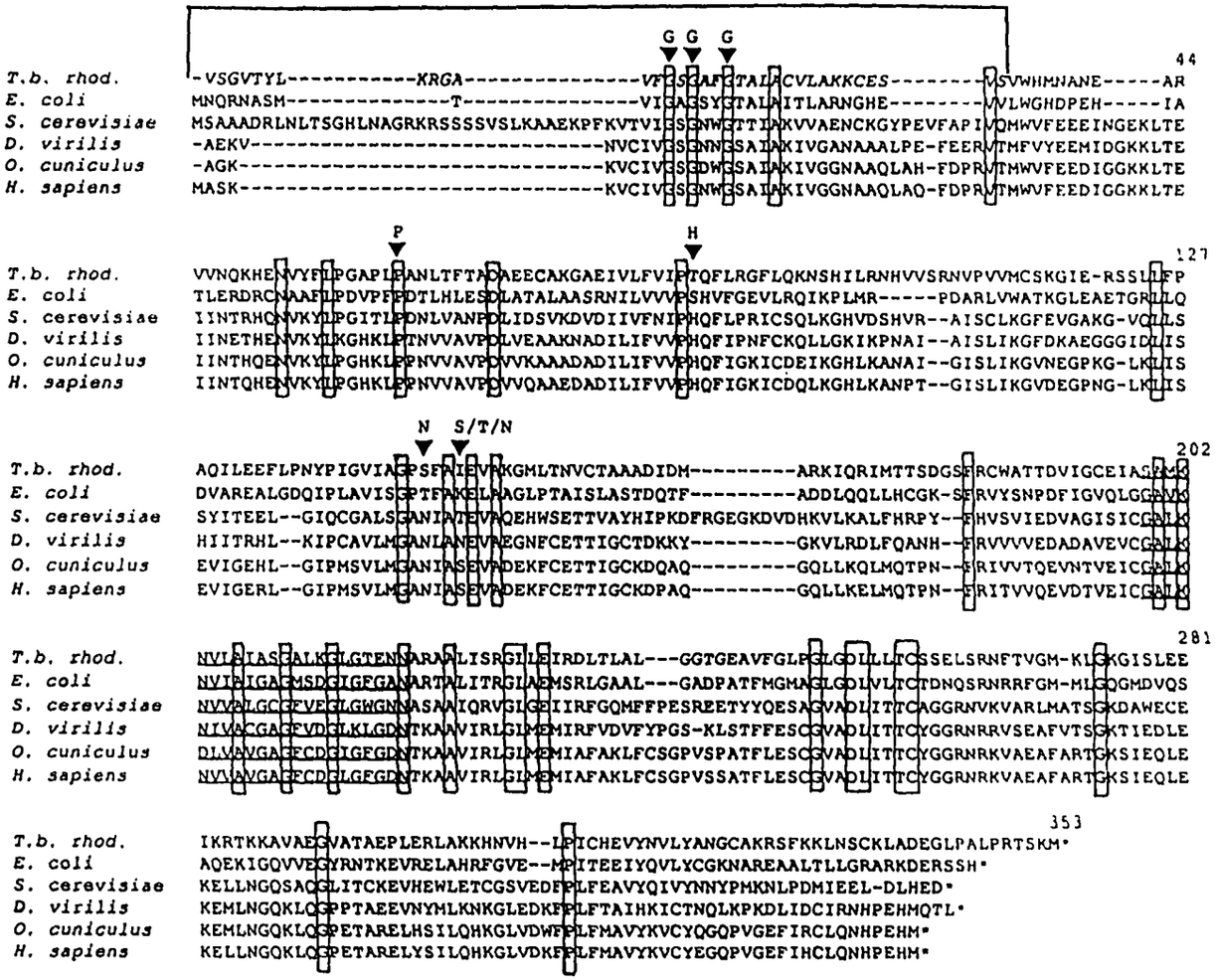

G G G
44
T.b. rhod. -VSGVTYL----------KRGA----------VFGSGAFGTALACVLAKKCES--------VSVWHMNANE-----AR
E. coli MNQRNASM----------T----------VIGAGSYGTALAITLARNGHE--------VVLWGHDPEH-----IA
S. cerevisiae MSAAADRLNLTSGHLNAGRKRSSSSVSLKAAEKPFKVTVIGSGNWGTTIAKVVAENCKGYPEVFAPIVQMWVFEEEINGEKLTE
D. virilis -AEKV----------NVCIVGSGNWGSAIAKIVGANAAALPE-FEERVTMFVYEEMIDGKKLTE
O. cuniculus -AGK----------KVCIVGSGDWGSAIAKIVGGNAAQLAH-FDPRVTMWVFEEDIGGKKLTE
H. sapiens MASK----------KVCIVGSGNWGSAIAKIVGGNAAQLAQ-FDPRVTMWVFEEDIGGKKLTE
P
H
127
T.b. rhod. VVNQKHENVYFTLPGAPLPANLTFTADAEECAKGAEIVLFVIPTQFLRGFLQKNSHILRNHVVSRNVPVVMCSKGIE-RSSLLFP
E. coli TLERDRCNAAFLPDVPFFDTLHLESDLATALAASRNILVVVPSHVFGEVLRQIKPLMR-----PDARLVWATKGLEAETGRLLQ
S. cerevisiae IINTRHQNVKYLPGITLPDNLVANPDLIDSVKDVDIIVFNIPHQFLPRICSQLKGHVDSHVR--AISCLKGFEVGAKG-VQLLS
D. virilis IINETHENVKYLKGHKLPTNVVAVPDLVEAAKNADILIFVVPHQFIPNFCKQLLGKIKPNAI--AISLIKGFDKAEGGGIDLIS
O. cuniculus IINTHQENVKYLPGHKLPPNVVAVPDVVKAAADADILIFVVPHQFIGKICDEIKGHLKANAI--GISLIKGVNEGPKG-LKLIS
H. sapiens IINTQHENVKYLPGHKLPPNVVAVPDVVQAAEDADILIFVVPHQFIGKICDQLKGHLKANPT--GISLIKGVDEGPNG-LKLIS
N S/T/N
202
T.b. rhod. AQILEEFLPNYPIGVIAGPSFAIEVVKGMLTNVCTAAADIDM---------ARKIQRIMTTSDGSFRCWATTDVIGCEIAGALKN
E. coli DVAREALGDQIPLAVISGPTFAKELAAGLPTAISLASTDQTF---------ADDLQQLLHCGK-SFRVYSNPDFIGVQLGGAVK
S. cerevisiae SYITEEL--GIQCGALSGANIATEVAQEHWSETTVAYHIPKDFRGEGKDVDHKVLKALFHRPY--FHVSVIEDVAGISICGALK
D. virilis HIITRHL--KIPCAVLMGANLANEVAEGNFCETTIGCTDKKY---------GKVLRDLFQANH--FRVVVVEDADAVEVCGALK
O. cuniculus EVIGEHL--GIPMSVLMGANIASEVADEKFCETTIGCKDQAQ---------GQLLKQLMQTPN--FRIVVTQEVNTVEICGALK
H. sapiens EVIGERL--GIPMSVLMGANIASEVADEKFCETTIGCKDPAQ---------GQLLKELMQTPN--FRITVVQEVDTVEICGALK
281
T.b. rhod. NVLAIASGALKGLGTENNARAALISRGLLEIRDLTLAL---GGTGEAVFGLPGLGDLLLTCSSELSRNFTVGM-KLGKGISLEE
E. coli NVIAIGAGMSDGIGFGANARTALITRGLAEMSRLGAAL---GADPATFMGMAGLGDLVLTCTDNQSRNRRFGM-MLGQGMDVQS
S. cerevisiae NVVALGCFEVEGLGHGNNASAAIQRVGLGEIIRFGQMFFPESREETYYQESAGVADLITTCAGGRNVKVARLMATSGKDAWECE
D. virilis NIVACGAFEVDGLKLGDNTKAAVIRLGLMEMIRFVDVFYPGS-KLSTFFESCGVADLITTCYGGRNRRVSEAFVTSGKTIEDLE
O. cuniculus DLVAVGAFECDGIGFGDNTKAAVIRLGLMEMIAFAKLFCSGPVSPATFLESCGVADLITTCYGGRNRKVAEAFARTGKSIEQLE
H. sapiens NVVAVGAFECDGLGFGDNTKAAVIRLGLMEMIAFAKLFCSGPVSSATFLESCGVADLITTCYGGRNRKVAEAFARTGKSIEQLE
353
T.b. rhod. IKRTKKAVAEGVATAEPLERLAKKHNVH--LPICHEVYNVLYANGCAKRSFKKLNSCKLADEGLPALPRTSKM*
E. coli AQEKIGQVVEGYRNTKEVRELAHRFGVE--MPITEEIYQVLYCGKNAREAALTLLGRARKDERSSH*
S. cerevisiae KELLNGQSACGLITCKEVHEWLETCGSVEDFPLFEAVYQIVYNNYPMKNLPDMIEEL-DLHED*
D. virilis KEMLNGQKLQGPPTAEEVNYMLKNKGLEDKFPLFTAIHKICTNQLKPKDLIDCIRNHPEHMQTL*
O. cuniculus KEMLNGQKLQGPETARELHSILQHKGLVDWFPLFMAVYKVCYQGQPVGEFIRCLQNHPEHM*
H. sapiens KELLNGQKLQGPETARELYSILQHKGLVDKFPLFMAVYKVCYEGQPVGEFIHCLQNHPEHM*

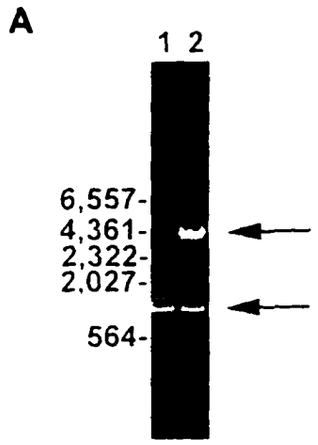
Table 5 Percentage identity between the amino acid sequences of glycerol 3-phosphate dehydrogenase from a variety of species. The percentages were calculated from the number of identical amino acid residues shared between the aligned sequences in Figure 7; in each case the percentage was calculated from the GPD amino acid sequence with the least number of residues.

	T.b. rhod. (protozoan)	*E. coli* (bacterium)	*S. cerevisiae* (yeast)	*D. virilis* (fruit fly)	*O. cuniculus* (rabbit)	*H. sapiens* (human)
T.b. rhod. (protozoan)	/	32.2 %	24.6 %	22.2 %	22.7 %	23.5 %
E. coli (bacterium)		/	24.8 %	22.4 %	24.8 %	24.2 %
S. cerevisiae (yeast)			/	46.9 %	46.0 %	48.1 %
D. virilis (fruit fly)				/	63.8 %	65.0 %
O. cuniculus (rabbit)					/	92.0 %
H. sapiens (human)						/

GPD are individually represented in Figure 7 [Otto et al., 1980]. Residues 14 to 19 contain the universally conserved Gly-X-Gly-X-X-Gly motif (where X denotes any amino acid) associated with the binding of the adenosine phosphate moiety of the NAD^+ coenzyme. Residue 62 (proline) has also been implicated in binding the coenzyme and this is conserved in all of the GPD enzymes compared. A histidine residue at position 94 of the rabbit GPD sequence has been suggested to be important in NAD^+-binding although this residue is absent from the trypanosome and *E. coli* sequences [Otto et al., 1980]. Residues presumed to participate in substrate binding include an asparagine at position 150 and either a serine, threonine or asparagine at position 153 of the rabbit GPD sequence [Otto et al., 1980]. Again, none of these were found in the trypanosome and *E. coli* GPD sequences. Figure 7 also shows the signature pattern characteristic of NAD^+-dependent GPD which was found using the Prosite™ protein motif database. The trypanosome sequence contains 17 of the 22 residues that constitute this signature pattern, while the other GPD sequences compared in Figure 7 are a perfect match to this consensus sequence.

Overexpression of the 38 kDa trypanosome protein in E. coli. Expression of the 38 kDa trypanosome protein as a fusion protein was accomplished by cloning the coding region into the pGEX-2T expression vector followed by subsequent expression in *E. coli*. Figure 8, panel A, lane 1 shows the PCR product amplified from *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA using PCR primers designed from the extreme termini of the 38 kDa protein coding region and engineered with restriction sites necessary for cloning into the pGEX-2T vector (Figure 8, panel A, lane 2). Figure 9, panel A shows a Coomassie blue stained SDS-PAGE gel of lysates of *E. coli* overexpressing the fusion protein. Lanes 1 and 2 show lysates of *E. coli* DH5α transformed with the pGEX-2T vector alone or with the pGEX-2T-putative GPD construct, respectively. The 64 kDa fusion protein (26 kDa GST protein plus 38 kDa trypanosome protein) observed in the DH5α/pGEX-2T-putative GPD lysate was absent from the DH5α/pGEX-2T lysate. To purify the fusion protein the bacterial lysate was passed over a glutathione-Sepharose 4B column and bound protein was eluted. Lanes 3 and 4 show protein profiles of the column eluates from the DH5α/pGEX-2T and DH5α/pGEX-2T-putative GPD lysates, respectively. The 64 kDa fusion protein was a major component of the eluate and was recovered in relatively large quantities. Thrombin cleavage of the fusion protein allowed recovery of a small amount of the 38 kDa trypanosome protein free of contaminating proteins (Lane 5). We were precluded from using this purified protein for subsequent manipulations as our yields after thrombin cleavage were too low. The fusion protein, when assayed for GPD

Figure 8 Agarose gel analyses of the amplified PCR fragments corresponding to the open reading frame encoding the 38 kDa protein (lower arrows) and the PCR fragments cloned into expression vectors (upper arrows). For visualization purposes the PCR fragments were excised from their respective expression vectors by digesting with the indicated restriction endonucleases. Molecular size standards (in base pairs) are indicated on the left of each panel. **A.** Lane 1, genomic PCR fragment. Lane 2, genomic PCR fragment cloned into the pGEX-2T vector (excised with *Bam*HI and *Eco*RI). **B.** Lane 1, genomic PCR fragment. Lane 2, genomic PCR fragment cloned into the pET-3d vector (excised with *Nco*I and *Bam*HI).


A
1 2
6,557-
4,361-
2,322-
2,027-
564-

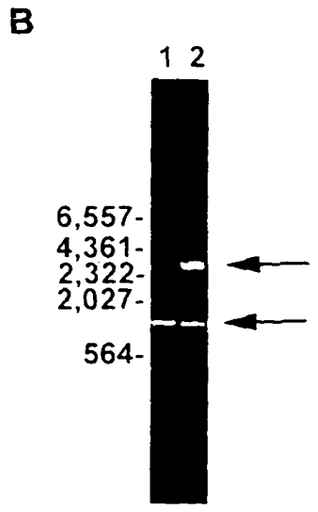
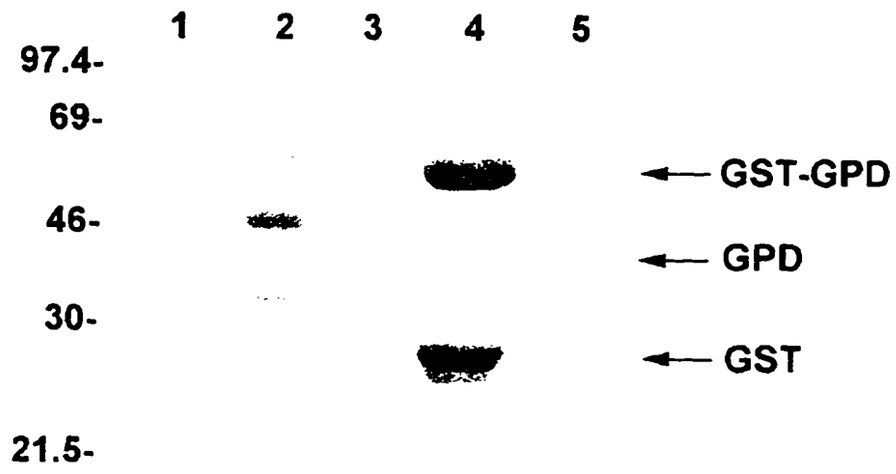

B
1 2
6,557-
4,361-
2,322-
2,027-
564-

Figure 9 SDS-PAGE and immunoblot analyses of GST fusion protein expression and purification. **A.** Coomassie blue stained gel. Lane 1, *E. coli* DH5α/pGEX-2T sonicate. Lane 2, *E. coli* DH5α/pGEX-2T-putative GPD sonicate. Lane 3, *E. coli* DH5α/pGEX-2T eluate from glutathione-Sepharose 4B. Lane 4, *E. coli* DH5α/pGEX-2T-putative GPD eluate from glutathione-Sepharose 4B. Lane 5, flow-through following thrombin digestion of GST fusion protein bound to glutathione-Sepharose 4B. **B.** Immunoblot analysis of SDS-PAGE separated proteins in *T.b. rhodesiense* ViTat 1.1 PCF whole cell lysate. In lane 1, mAb 6A9 tissue culture supernatant was used as primary antibody at a 1:1 dilution. Protein molecular mass markers (in kilodaltons) are indicated on the left of both panels.

A



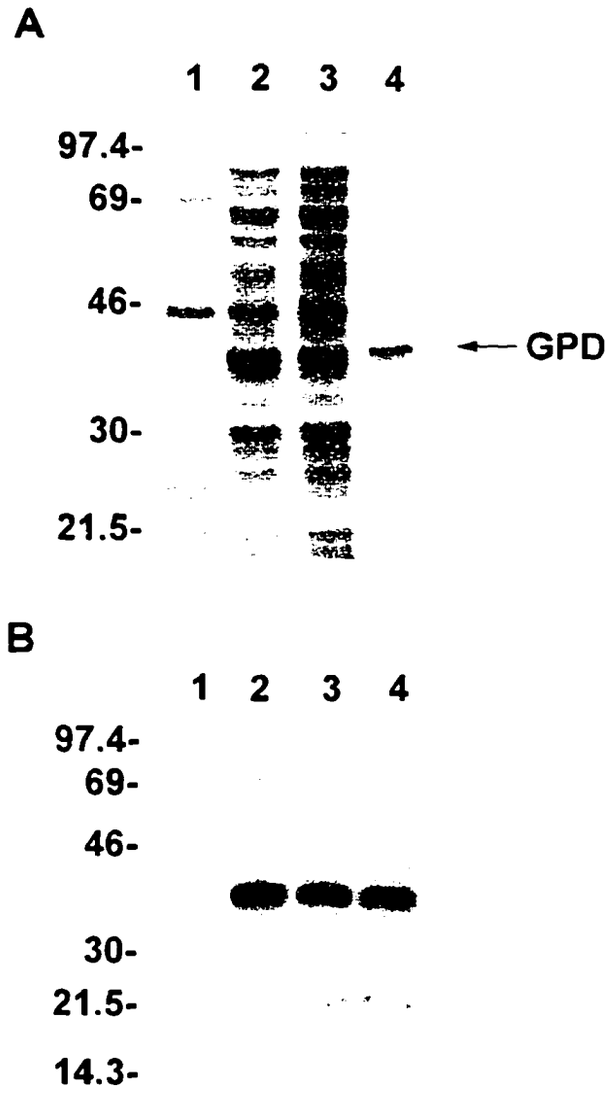
B

enzyme activity, was found to exhibit a specific activity of 9.66 units mg^{-1}. Although this activity is low compared with other glycosomal enzymes [Kohl et al., 1994; Hannaert et al., 1994], possibly due to interference from the GST portion of the fusion protein, the activity does verify the identity of the overexpressed protein as an NAD^+-dependent GPD.

The GST-GPD fusion protein was used as immunogen to produce 30 mAbs specific for the 38 kDa trypanosome GPD. *T.b. rhodesiense* ViTat 1.1 PCF lysates were subjected to SDS-PAGE and the separated proteins were tested in immunoblots with 10 mAbs that were shown to exhibit strong binding to the GPD portion of the fusion protein in ELISA. One of the mAbs, 6A9, revealed a strongly immunoreactive band corresponding to the trypanosome GPD at 38 kDa (Figure 9, panel B, lane 1).

The 38 kDa molecule was also expressed in *E. coli* as a recombinant, non-fusion protein using the pET-3d overexpression vector. Figure 8, panel B, lane 1 shows the PCR product amplified from *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA using PCR primers designed from the extreme termini of the 38 kDa protein coding region and engineered with restriction sites necessary for cloning into the pET-3d vector (Figure 8, panel B, lane 2). Figure 10, panel A is a Coomassie blue stained SDS-PAGE gel showing the results of the overexpression studies. Lanes 1 and 2 show lysates of *E. coli* BL21(DE3)pLysS transformed with the pET-3d vector alone or with the pET-3d-GPD construct, respectively. The 38 kDa recombinant protein observed in the BL21(DE3)pLysS/pET-3d-GPD lysate was absent from the BL21(DE3)pLysS/pET-3d lysate. The recombinant overexpressed protein was partially purified by preparing crude soluble and insoluble fractions from the BL21(DE3)pLysS/pET-3d-GPD lysate, and the 38 kDa recombinant protein was found (by SDS-PAGE analysis) in both fractions (Figure 10, panel A, lanes 3 and 4, respectively). The identity of the overexpressed 38 kDa recombinant protein as the trypanosome GPD was verified by immunoblot analysis of the overexpression lysates and fractions. An SDS-PAGE gel identical to the one described in Figure 10, panel A was prepared and the proteins were electrophoretically transferred to Immobilon-P™ and immunologically probed with the GPD-specific mAb 6A9 (Figure 10, panel B, lanes 1-4). A 38 kDa immunoreactive protein was observed in the BL21(DE3)pLysS/pET-3d-GPD lysate (lane 2) and in both the soluble and insoluble fractions of the lysate (lanes 3 and 4, respectively), but was not observed in the BL21(DE3)pLysS/pET-3d lysate (lane 1). The BL21(DE3)pLysS/pET-3d/GPD lysate was assayed for GPD enzyme activity and was found to be enzymatically very active (data not shown).

Figure 10 SDS-PAGE and immunoblot analyses of recombinant, non-fusion GPD protein expression and its partial purification. Protein molecular mass markers (in kilodaltons) are indicated on the left of each panel. **A.** An SDS-PAGE gel was stained with Coomassie blue. Lane 1, *E. coli* BL21(DE3)pLysS/pET-3d lysate. Lane 2, *E. coli* BL21(DE3)pLysS/pET-3d-GPD lysate. Lane 3, *E. coli* BL21(DE3)pLysS/pET-3d-GPD crude soluble fraction. Lane 4, *E. coli* BL21(DE3)pLysS/pET-3d-GPD crude insoluble fraction. **B.** Immunoblot analysis. Lane assignments are as outlined in panel A. MAb 6A9 tissue culture supernatant was used as primary antibody at a 1:1 dilution.


A
1 2 3 4
97.4-
69-
46-
30-
21.5-
GPD
B
1 2 3 4
97.4-
69-
46-
30-
21.5-
14.3-

Kinetic analyses of recombinant non-fusion GPD and rabbit muscle GPD gave Km values for dihydroxyacetone phosphate of 0.646 ± 0.081 mM and 0.320 ± 0.065 mM, respectively, thus showing the trypanosome enzyme differs from its mammalian counterpart (n=4, $p < 0.02$, t-test).

GPD localization. The localization of mAb 6A9 reactivity, and thus GPD localization, was investigated by immunofluorescence and immunogold electron microscopy. Immunofluorescence on acetone-permeabilized *T.b. brucei* 427 PCF showed strong fluorescence localized to the glycosomes of the parasite (data not shown). Immunoelectron microscopy on *T.b. brucei* 427 BSF (A) and PCF (B) showed a glycosomal localization of the protein A gold particles (Figure 11). The localization of GPD within the glycosome was further verified by mAb 6A9 immunoblot analysis of glycosomes purified from *T.b. rhodesiense* ViTat 1.1 PCF. Figure 12, lane 1 shows the Coomassie blue stained SDS-PAGE glycosomal protein profile. A 38 kDa protein band was visible in the glycosome preparation. Immunoblot analysis identified the 38 kDa GPD in the glycosome lysates (Figure 12, lane 2).

Immunological studies. The species and life cycle stage distribution of the GPD was analyzed by immunoblotting using mAb 6A9. As shown in Figure 13, a 38 kDa immunoreactive band was observed in approximately equal amounts in all of the African trypanosome species and subspecies tested (*T.b. rhodesiense*, *T.b. brucei*, *T. simiae* and *T. congolense*, panel A, lanes 1-4, respectively), and in all of the life cycle stages examined (PCF from *T.b. rhodesiense*, *T.b. brucei*, *T. simiae* and *T. congolense* IL3000, panels A and B lane 1, panel A lane 2, panel A lane 3, and panels A and B lane 4, respectively; BSF from *T.b. rhodesiense* and *T. congolense* IL3000, panel B, lanes 2 and 3, respectively; epimastigotes and metacyclics from *T. congolense* IL3000, panel B, lanes 5 and 6, respectively). GPD expression during transformation of *T. congolense* IL3000 from BSF to PCF was also investigated, and GPD was found to be expressed at approximately equal levels throughout the 72 h transformation experiment (Figure 13, panel C). Immunoreactivity was not found in other members of the kinetoplastid family, as shown by the absence of mAb 6A9 immunoreactivity against *L. donovani* LD3, *L. major* 5-ASKH and *L. tropica* K27.3 promastigotes, *T. cruzi* Peru strain epimastigotes, Y strain trypomastigotes and G strain amastigotes and *Crithidia fasciculata* 11745 (data not shown).

Figure 11 Immunoelectron microscopy of Lowicryl K4M-embedded *T.b. brucei* labeled with GPD-specific mAb 6A9. Both BSF (**A**) and PCF (**B**) were labeled by mAb 6A9 and 10 nm protein A gold. F = flagellum, FP = flagellar pocket, N = nucleus, K = kinetoplast, M = mitochondrion. Bars = 1mm.

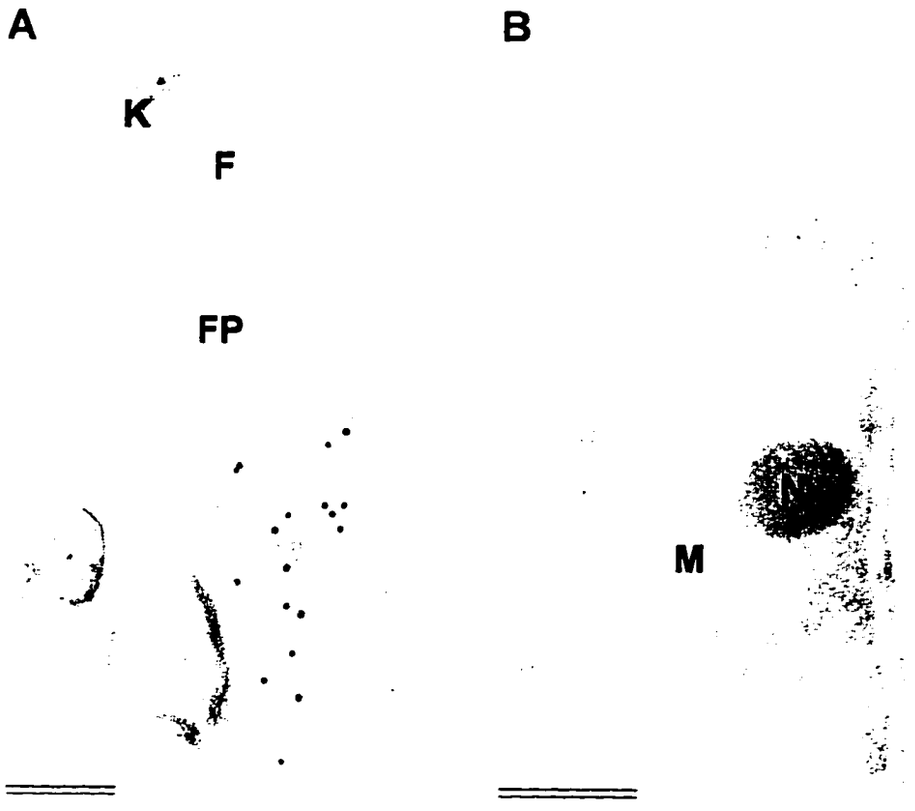

A
K
F
FP
B
M

Figure 12 SDS-PAGE and immunoblot analyses of *T.b. rhodesiense* ViTat 1.1 PCF purified glycosomes. Protein molecular mass markers (in kilodaltons) are indicated on the left. Lane 1, SDS-PAGE-separated glycosomal proteins, stained with Coomassie blue. Lane 2, immunoblot analysis of SDS-PAGE-separated glycosomal proteins. Tissue culture supernatant containing mAb 6A9 was used at a 1:1 dilution.

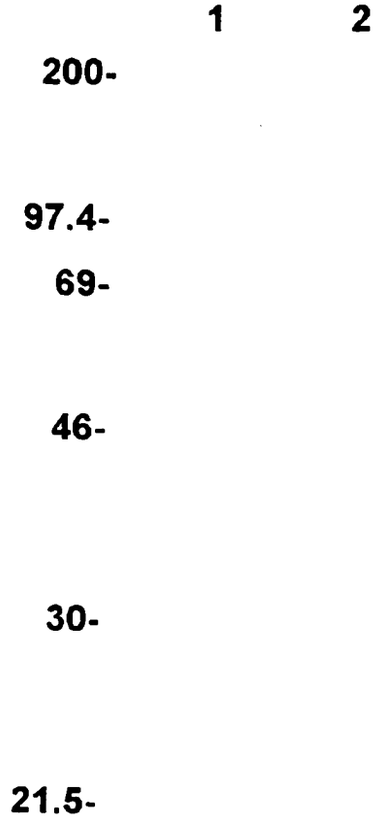
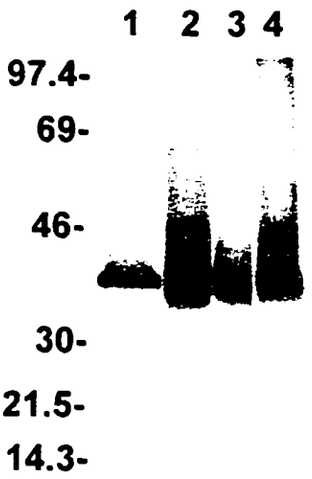

1
2
200-
97.4-
69-
46-
30-
21.5-

Figure 13 Immunoblot analyses of the species and life cycle stage distribution of trypanosome GPD. Protein molecular mass markers (in kilodaltons) are indicated on the left of each panel. Tissue culture supernatant containing mAb 6A9 was used at a 1:1 dilution. **A.** Expression of GPD in various species and subspecies of African trypanosomes. Lane 1, *T.b. rhodesiense* ViTat 1.1 PCF. Lane 2, *T.b. brucei* 427.01 PCF. Lane 3, *T. simiae* CP-11 PCF. Lane 4, *T. congolense* IL3000 PCF. **B.** Expression of GPD throughout the trypanosome life cycle. Lane 1, *T.b. rhodesiense* ViTat 1.1 PCF. Lane 2, *T.b. rhodesiense* ViTat 1.1 BSF. Lane 3, *T. congolense* IL3000 BSF. Lane 4, *T. congolense* IL3000 PCF. Lane 5, *T. congolense* IL3000 epimastigotes. Lane 6, *T. congolense* IL3000 metacyclics. **C.** Expression of GPD during transformation from BSF to PCF. Lane assignments correspond to the intervals (in hours) at which samples were taken for immunoblot analysis.

A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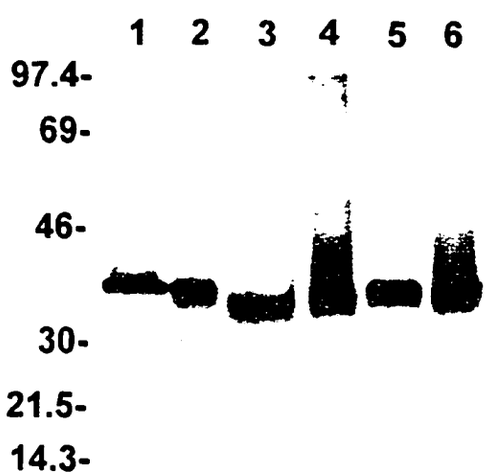
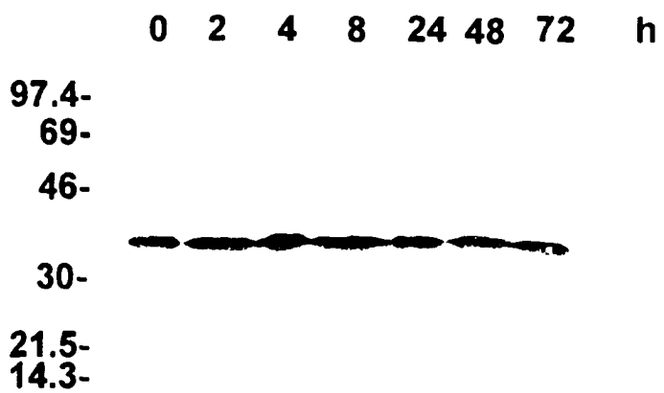
14.3-

B


1 2 3 4 5 6
97.4-
69-
46-
30-
21.5-
14.3-

C


0 2 4 8 24 48 72 h
97.4-
69-
46-
30-
21.5-
14.3-

GPD gene copy number and mRNA transcript characterization. The GPD gene copy number was determined by Southern blot analysis of restriction endonuclease digests of *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA probed with the homologous trypanosome genomic GPD probe. A variety of restriction endonucleases were selected for this analysis, including three which contained cleavage sites within the GPD gene (*Kpn*I, *Nde*I, *Sau*3AI), and four for which cleavage sites were not found within the gene (*Bam*HI, *Eco*RI, *Hind*III, *Xho*I). Figure 14, panel A is a diagram of the restriction endonuclease map for the GPD gene. As shown in Figure 14, panel B the *Nde*I digest showed four hybridizing DNA fragments (lane 5), the *Kpn*I and *Sau*3AI digests each showed three hybridizing fragments (lanes 4 and 6, respectively), the *Eco*RI and *Xho*I digests each showed two hybridizing fragments (lanes 2 and 7, respectively) and the *Bam*HI and *Hind*III digests each showed one hybridizing fragment (lanes 1 and 3, respectively). These results suggest the presence of at least two gene copies encoding the trypanosome NAD^+-dependent GPD. The homologous GPD gene was also used as a probe in Northern blot analysis and was found to hybridize to a single GPD mRNA transcript of approximately 1,620 bases (Figure 14, panel C).

Bacteriophage P1 library screen. To obtain genomic DNA sequence flanking the GPD gene open reading frame, a bacteriophage P1 library high density filter representative of the leishmania and trypanosome genomes was screened with the trypanosome genomic GPD probe. The filter colony layout is shown in Figure 15. The screen identified numerous clones that exhibited reactivity with the homologous probe, and five were selected for further analysis (Figure 16; selected positives indicated by arrows). DNA was isolated from each of the five positive clones and rescreened by dot blotting methodology to verify positivity. One positive clone was selected, and its isolated DNA was subjected to restriction endonuclease digestion and reprobed by Southern blotting with the homologous GPD probe. One well-isolated hybridizing DNA fragment of approximately 4,500 bp was selected (data not shown). This fragment size is substantially larger than the GPD open reading frame (1062 base pairs), and thus will probably contain large stretches of genomic flanking sequences that can be used to design plasmid constructs for knockout mutagenesis (this work is currently in progress) and is beyond the scope of this thesis.

Figure 14 Analysis of *T.b. rhodesiense* GPD gene copy number and expression of the mRNA transcript by Southern and Northern blots. **A.** Restriction endonuclease map for the 1065 bp trypanosome GPD coding region. Only restriction sites used in the Southern blot analysis are indicated. **B.** Southern blot analysis of *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA probed with the homologous GPD gene. Lanes 1 to 7 represent genomic DNA digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Nde*I, *Sau*3AI and *Xho*I, respectively. **C.** Northern blot analysis of *T.b. rhodesiense* ViTat 1.1 PCF poly(A)$^+$ RNA probed with the homologous GPD gene. Molecular size standards (in base pairs) are indicated on the left of each panel.

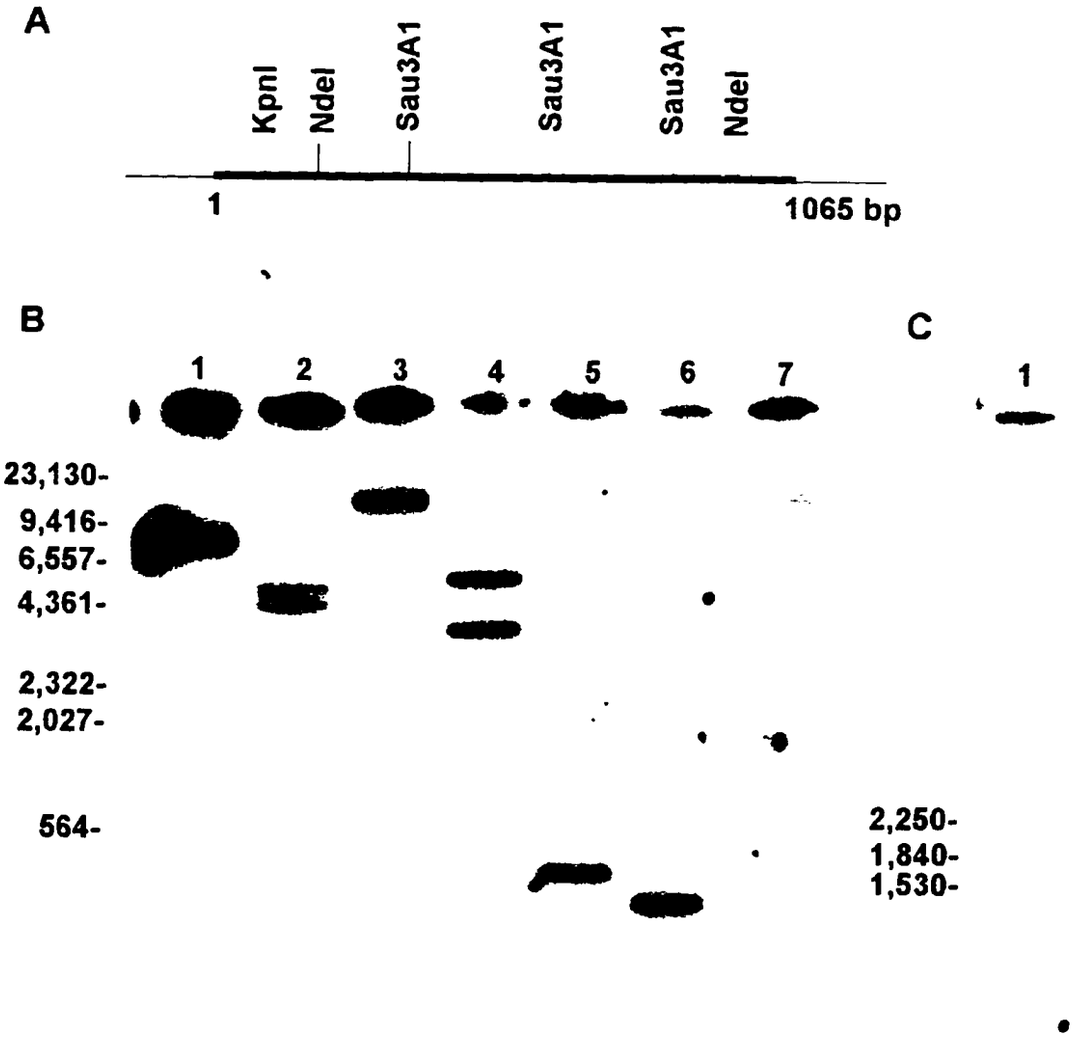
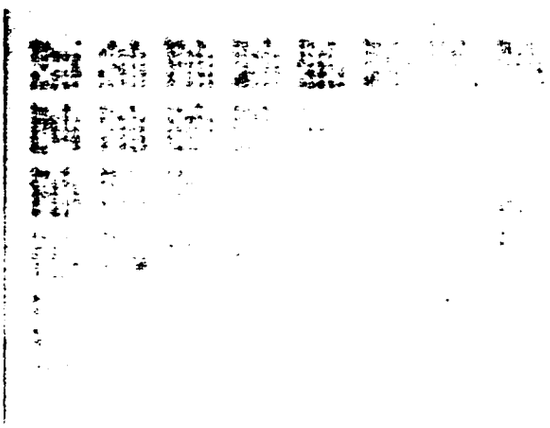

A
KpnI
NdeI
Sau3A1
Sau3A1
Sau3A1
NdeI
1
1065 bp
B
1
2
3
4
5
6
7
23,130-
9,416-
6,557-
4,361-
2,322-
2,027-
564-
C
1
2,250-
1,840-
1,530-

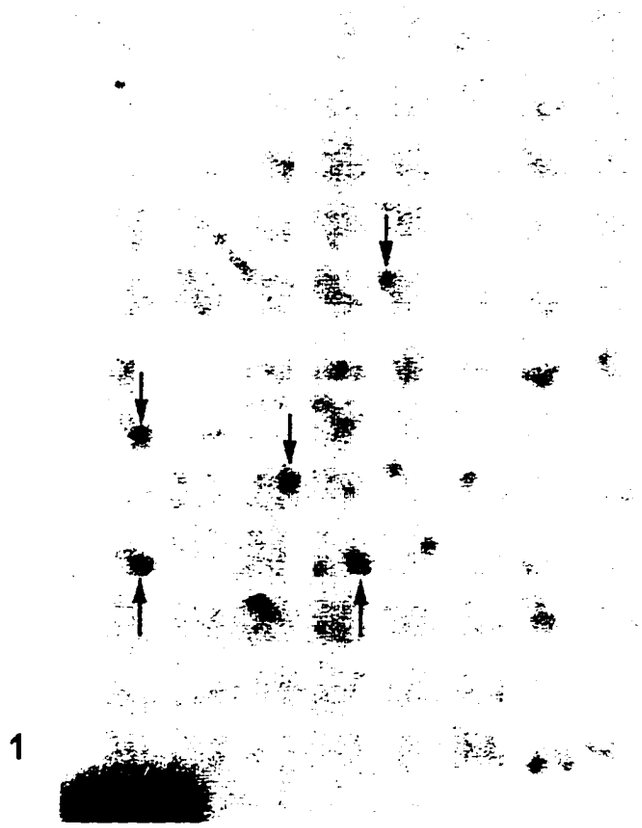
Figure 15 High density filter colony layout of the bacteriophage P1 trypanosome/leishmania library. To facilitate identification of hybridizing clones the filter is oriented such that the letters A to H extend from left to right and the numbers 1 to 12 extend from bottom to top (for simplicity only the letter A and the number 1 are indicated on the diagram).



1

A

Figure 16 Autoradiograph of the bacteriophage P1 trypanosome/leishmania library high density filter probed with the 1065 bp GPD gene from *T.b. rhodesiense* ViTat 1.1 PCF. The hybridizing clones that were selected for further study are indicated by arrows. The filter orientation is as described in Figure 15.


1
A

DISCUSSION

This chapter reports the molecular characterization of a 38 kDa protein purified from *T.b. rhodesiense* plasma membrane-enriched preparations and analysis of its encoding gene. This protein was repeatedly found to incorporate label when procyclic trypanosomes were subjected to mild, cell surface labeling analyses using a variety of reagents, including ^{125}Iodine, sulfo-NHS-biotin and NHS-SS-biotin (possible reasons for such label incorporation are discussed in detail below). The DNA sequence encoding the trypanosomal 38 kDa protein was determined by sequential rounds of PCR amplifications from both complementary and genomic DNA with subsequent DNA sequencing of the amplified fragments. The resultant translated protein was found to have a calculated molecular mass of 37,745 Da, an unusually high net positive charge of +9 and a correspondingly high pI of 9.0. Comparative analysis of the translated amino acid sequence with the protein database revealed a low but significant level of sequence similarity with NAD^+-dependent glycerol 3-phosphate dehydrogenase from various prokaryotic and eukaryotic organisms. In addition, the signature sequence pattern contained within the Prosite™ protein motif database for NAD^+-dependent GPD was found to be largely conserved within the *T.b. rhodesiense* 38 kDa protein.

Closer examination of the trypanosomal 38 kDa protein revealed evidence supporting its identity as the NAD^+-dependent GPD. Previous investigations had localized NAD^+-dependent GPD-specific enzyme activity to the trypanosome glycosome [Opperdoes and Borst, 1977; Opperdoes et al., 1977; Hart et al., 1984]. The localization of the trypanosomal 38 kDa protein to this organelle was immunologically confirmed using mAb 6A9 as a probe in immunofluorescence and immunogold electron microscopy analyses, and this localization was further verified by immunoblotting on proteins of purified glycosomes. It is interesting that the majority of glycosomal enzymes that have been described in *T. brucei* spp. have an unusually high net positive charge, which translates into a characteristically high pI (1-4 pH units higher), and a larger molecular mass (up to 5 kDa) when compared to the corresponding mammalian enzymes [Misset et al., 1986; Barnard et al., 1993]. The 38 kDa trypanosome molecule matches this pattern in being 2.0 pH units (13 positive charges) higher and 0.2 kDa larger than mammalian GPD [Otto et al., 1980]. Direct evidence that the 38 kDa protein is a trypanosome GPD was obtained by showing specific enzymatic activity of both the GST fusion protein and the non-fusion protein. These data collectively confirm the identity of the 38 kDa trypanosome protein as a glycosomal NAD^+-dependent GPD.

The repeated cell surface labeling of the trypanosome GPD and its isolation from preparations enriched in plasma membranes is suggestive of a cell surface disposition for this molecule. Although these results appear to diametrically oppose the glycosomal localization clearly demonstrated for this protein, several plausible explanations accounting for these unusual results can be posed. (1) It has been well documented that the trypanosomal glycosomal enzymes glyceraldehyde 3-phosphate dehydrogenase and adenylate kinase exhibit an affinity for the glycosomal membrane [Barnard et al., 1993; McLaughlin, 1985b]. In addition, 30% of the glycosomal protein content has been shown to localize to the organellar membrane, while the remaining proteins localize to the matrix [Opperdoes et al., 1984]. Therefore, the membrane affinity existing for the GPD may represent a true interaction of this enzyme with the glycosomal membrane, and contamination of plasma membranes with other organellar membranes could have lead to the isolation of GPD from such preparations. The simultaneous isolation of glyceraldehyde 3-phosphate dehydrogenase from these preparations (refer to result section) lends further support to this theory. Whether or not any of the glycosomal enzymes, including the GPD, are associated with the glycosomal membrane *in vivo* is not known. The incorporation of surface label into GPD under such circumstances could only be explained by cell disruption during the labeling process with subsequent label incorporation. Although surface labeling was performed under mild physiological conditions and cell viability was judged to be unaffected throughout, a small number of trypanosomes could have lysed, releasing the rather abundant GPD enzyme which could then become labeled. (2) A second possible explanation is that protein-protein interactions due to the highly positively charged nature of the glycosomal GPD could lead to membrane association upon cell lysis. In this scenario surface label incorporation would result from random cell lysis within the general parasite population during the labeling interval, while co-isolation with membrane preparations would occur during the cell lysis step in preparation of plasma membranes. In this vein it is interesting that α-tubulin (band 1 in Figure 3 and Table 4), a cytoskeletal protein found in close apposition to the overlying plasma membrane [Seebeck et al., 1988], also incorporated label and was isolated from the plasma membrane-enriched fractions. Previous reports have described the artifactual copurification of cytoskeletal proteins with glycosomal enzymes [Balaban and Goldman, 1990], thus indicating that a variety of protein-protein interactions may be occurring during cell lysis and protein isolation. (3) A third theory stems from the assumption that two distinct 38 kDa proteins exist, one of unknown identity associated with the plasma membrane and the second the glycosomal membrane-localized GPD. In

this theory the unknown 38 kDa plasma membrane protein would incorporate label, while the glycosomal GPD from which the NH_2-terminal sequence was obtained would co-isolate with the plasma membrane fraction. One complicating factor relevant to this theory is the attainment of the NH_2-terminal amino acid sequence from the biotinylation/avidin-agarose chromatography experiments that exactly matched the GPD sequence obtained from the plasma membrane-enriched fraction. The only possible rationalization for this phenomenon would be, once again, that the glycosomal GPD incorporated label through random parasite lysis during the period of cell surface labeling. If it was present in excess of any other 38 kDa protein or if the unknown 38 kDa protein was blocked at its NH_2-terminus, then the GPD sequence would be the only one detected. (4) A fourth possibility is that a GPD isoenzyme is actually present on the trypanosome plasma membrane. Another NAD^+-utilizing enzyme (dihydrolipoamide dehydrogenase) normally associated with an organelle involved in bioenergetics is found on the surface of African trypanosomes. This enzyme is normally a component of such mitochondrial multi-enzyme complexes as 2-oxoglutarate dehydrogenase and is clearly found on the surface of *T. brucei* BSF [Danson et al., 1987; Jackman et al., 1990]; there is no known function for this dihydrolipoamide dehydrogenase.

The true relationship of the NAD^+-dependent GPD to the plasma membrane must await further studies. However, if this enzyme is in fact localized to the cell surface the questions arises as to its functional relevance to the parasite. The answer may relate to the observation that intact BSF respire actively with glycerol 3-phosphate[6], a substrate that would not be expected to be taken up by intact cells. Thus, a surface localized NAD^+-dependent GPD that could in some way be involved in metabolism of glycerol substrates at the cell surface may be yet another example of the uniqueness of trypanosomes compared to other eukaryotic organisms.

Immunoblot analyses using mAb 6A9 showed that the 38 kDa GPD molecule was expressed in all species and subspecies of African trypanosomes tested (*T.b. rhodesiense*, *T.b. brucei*, *T. simiae* and *T. congolense*) and was reactive against both bloodstream and insect (procyclic, epimastigote and metacyclic) life cycle stages. In all instances the level of GPD detected appeared approximately equal. GPD was also found to be expressed at approximately equal levels throughout transformation of *T. congolense* BSF to PCF.

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[6] Clarkson, A. B. Jr. and Bienen, J., Personal communication.

These findings correlate with a previous report stating GPD-specific activity remains constant during transformation from BSF to PCF [Opperdoes, 1987]. Although the GPD protein level and enzyme activity remains constant between BSF and PCF trypanosomes, the metabolic role of this enzyme varies between the two life cycle stages; as the glycerol 3-phosphate shuttle is inoperative in PCF trypanosomes [Opperdoes, 1987], the metabolic emphasis of GPD shifts to its role in glycerol metabolism. Further analyses detected no mAb 6A9 reactivity in other kinetoplastid parasites, including *Leishmania*, *Trypanosoma cruzi* and *Crithidia*, thus indicating a certain amount of sequence divergence in GPD molecules from these related protozoans. This suspected sequence divergence has recently been confirmed in the case of the leishmanial GPD, which has been found to exhibit 63% sequence similarity with the trypanosomal GPD [Kohl et al., 1996].

Previous reports have described the purification of the FAD-dependent GPD from the mitochondrial membrane of *T. brucei* BSF [Kornblatt et al., 1992]. The degree of sequence similarity between the glycosomal NAD^+-dependent GPD described in this chapter and its FAD-dependent mitochondrial counterpart must await the elucidation of the primary sequence of the FAD-dependent GPD. However, several lines of evidence suggest these represent two distinct enzymes with significant sequence divergence. First, the most obvious distinction existing between the two enzymes is their vast size difference (38 vs 67 kDa for the NAD^+- and FAD-dependent GPD, respectively) [Kornblatt et al., 1992]. Second, the observation that only one PCR product was amplified using primers designed from either the GPD NH_2-terminal amino acid sequence or from the extreme termini of the GPD coding region demonstrates that at least those portions of the enzyme sequences differ between the two cellular locations. Third, the immunolocalization of mAb 6A9 to the glycosome and not to the mitochondrion further verifies the uniqueness of the two enzymes. Comparative analyses of NAD^+- and FAD-dependent GPD enzymes from *S. cerevisiae* and *E. coli* reveal minimal amino acid sequence similarity (13% and 14%, respectively) [Rennow and Kielland-Brandt, 1993; Weiner, 1988], suggesting that this may also be the case with the trypanosome enzymes.

Glycosomal glycolytic enzymes that have been characterized at the molecular level include glucosephosphate isomerase [Marchand et al., 1989], fructose-bisphosphate aldolase [Clayton, 1985], triosephosphate isomerase [Swinkels et al., 1986], glyceraldehyde 3-phosphate dehydrogenase [Michels et al., 1986], phosphoglycerate kinase [Osinga et al., 1985] and glycerol 3-phosphate dehydrogenase [this report; Kohl et al., 1996]. In two instances (glyceraldehyde 3-phosphate dehydrogenase and

phosphoglycerate kinase), cytosolic isoenzymes have been found for these glycosomal enzymes. These cytosolic isoenzymes exhibit 55% and 95% sequence similarity, respectively, with their glycosomal counterparts [Michels et al., 1986; Osinga et al., 1985]. The glycosomal phosphoglycerate kinase isoenzyme exhibits high activity in BSF trypanosomes and minimal activity in PCF trypanosomes, while the reverse is observed for the cytosolic isoenzyme [Parsons and Hill, 1989]. The glycosomal glyceraldehyde 3-phosphate dehydrogenase accounts for 80% of the total enzyme activity in both BSF and PCF trypanosomes [Misset et al., 1987].

Earlier investigations have suggested both the presence and absence in trypanosomes of a cytosolic NAD^+-dependent GPD which acts to convert dihydroxyacetone phosphate to glycerol 3-phosphate and regenerate NAD^+ in the cytosol. The prediction of the existence of such an enzyme was based solely on its presence in other eukaryotes and has not yet been confirmed [Kornblatt et al., 1992]. More compelling evidence for the absence of such an isoenzyme was based upon studies showing the presence of only one GPD-specific enzyme activity peak in hydrophobic interaction chromatography fractions of trypanosome soluble extracts, while two were observed for glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase [Misset et al., 1987]. Although the presence of such a cytosolic isoenzyme was not investigated in these studies, the lack of cytosolic immunoreactivity of mAb 6A9 in immunogold electron microscopy and immunofluorescence and similar results obtained using an antiserum specific for the glycosomal GPD[7] suggest that an immunologically cross-reactive GPD isoenzyme may not exist. However, the presence of a cytosolic GPD isoenzyme would not in any way diminish the importance of the glycosomal enzyme, since the glycosomal membrane is impermeable to NAD^+ [Borst, 1977] and thus this organelle would still require an internal mechanism for its regeneration.

Glycosomal enzymes are synthesized on free ribosomes in the cytosol and are imported into the glycosome post-translationally without any form of processing or secondary modification [Hart et al., 1987]. It has been shown that a C-terminal SKL-like tripeptide involved in protein import into peroxisomes functions in the import of proteins into the glycosomes of trypanosomes [Blattner et al., 1992; Sommer and Wang, 1994]. Glyceraldehyde 3-phosphate dehydrogenase, glucosephosphate isomerase,

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[7] Stebeck, C. E. and Pearson, T. W., Unpublished results.

phosphoglycerate kinase and phosphoenolpyruvate carboxykinase have been shown to utilize AKL, SHL, SSL and SRL tripeptide sequences, respectively, for glycosomal import [Sommer and Wang, 1994; Marchand et al., 1989; Michels et al., 1986; Sommer et al., 1993; Sommer et al., 1994]. The *T.b. rhodesiense* GPD reported in this chapter, as well as the glyceraldehyde 3-phosphate dehydrogenase from the related kinetoplastid *Leishmania mexicana*, contain a C-terminal tripeptide sequence SKM which also functions as a glycosomal targeting signal sequence [Sommer and Wang, 1994]. Alternative targeting signals must exist as well for glycosomal enzymes such as triosephosphate isomerase and aldolase [Clayton, 1985; Allert et al., 1991] for which no such tripeptide sequence is present.

Trypanosome glycosomal enzymes have generally been found to exhibit a lower degree of identity with enzymes from other organisms than do the enzymes of *E. coli* and yeast or other eukaryotes [Michels et al., 1986]. However, the percentage identity between the NAD^+-dependent GPD of *T.b. rhodesiense* and GPD of other organisms is unusually low. The highest degree of sequence identity is with *E. coli* (32.2%), while identity with GPD from eukaryotes is lower (22.2-24.6%). The *T.b. rhodesiense* GPD is unusual in this respect, as the majority of other glycosomal enzymes from *T. brucei* more closely resemble their eukaryotic counterparts (45-58%) [Swinkels et al., 1986].

Although there is not yet a crystal structure for GPD of any species, secondary structure analysis of rabbit GPD and comparisons with other dehydrogenases for which the tertiary structures are known have identified amino acids presumed to be essential for enzyme catalysis [Otto et al., 1980]. The Gly-X-Gly-X-X-Gly motif at positions 14 to 19 and the Pro-62 residue implicated in NAD^+-binding are found in the trypanosome sequence; however, neither the His-94 residue implicated in binding the coenzyme nor the residues determined to be important in substrate binding are present. This is in direct contrast to the high degree of sequence conservation of active site residues observed between several other trypanosome glycosomal enzymes and their mammalian counterparts [Michels et al., 1986; Swinkels et al., 1986]. In addition, the Km for the recombinant *T.b. rhodesiense* GPD was markedly different from that of the mammalian enzyme (Km of 0.646 ± 0.081 mM vs. 0.320 ± 0.065 mM, respectively). Taken together, the different Km values, the lack of sequence similarity and differences in the presumed NAD^+-binding site and substrate active site residues for the mammalian and trypanosomal GPD enzymes, as well as the functional relevance of GPD to the

bloodstream trypanosome, suggest that this protein may be a potential target for design of trypanocidal drugs that will have minimal effect on the mammalian enzyme.

The expression reported in this chapter of the trypanosome NAD^+-dependent GPD as a recombinant, non-fusion protein represents the first step in our ultimate goal of crystallizing this glycosomal enzyme. Current studies are focusing on purification of this recombinant protein to a state suitable for crystallization. The recombinant GPD was found to partition into both the soluble and insoluble fractions of the *E. coli* overexpression lysate; this presence of the recombinant protein in the soluble fraction will greatly simplify its purification.

Attainment of the trypanosome NAD^+-dependent GPD crystal structure will facilitate the design of GPD-specific inhibitors. To achieve favorable inhibition the drug must preferentially interact with the trypanosomal enzyme, a feat which may be enhanced by the highly positively charged nature of the trypanosome GPD. All glycosomal enzymes, with the exception of glucosephosphate isomerase, have unusually high isoelectric points within the range 8.8 to 10.2 [Misset et al., 1986; Opperdoes, 1987]. Modeling of the three-dimensional structures of trypanosomal triosephosphate isomerase, phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase using the known three-dimensional structures of homologous enzymes from other organisms indicates a clustering of positive charges into two areas approximately 40 A° apart on the enzyme's surfaces [Wierenga et al., 1987]. This charge clustering is not observed in the cytosolic isoenzymes of glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase, nor in the glycolytic enzymes of mammals or yeast, all of which have significantly lower pI values (1 to 6 pH units lower) [Opperdoes, 1987]. The widely used trypanocidal drug Suramin, which has been shown to inhibit the majority of glycosomal, glycolytic enzymes, is thought to partially utilize this glycosomal enzyme-specific predominance of positive charges in its mechanism of action. This drug possesses six negatively charged sulphonyl groups, and it is suggested that the positive charges on the enzymes' surfaces play an important role in binding of the negatively charged Suramin molecule. Not surprisingly, glucosephosphate isomerase, which possesses neither a high isoelectric point nor a clustering of positive charges, is not inhibited by Suramin. This preference for basic proteins is further exemplified by the low affinity of Suramin for the cytosolic isoenzymes glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase and for the glycolytic enzymes of mammals and yeast [Misset and Opperdoes, 1987]. The enzymes involved in the glycerol 3-

phosphate shuttle are the only enzymes inhibited by low concentrations of Suramin [Fairlamb et al., 1977], suggesting that these enzymes have a similar clustering of positive charges. Therefore, design of a negatively charged GPD inhibitor may serve to potentiate binding and thus inhibition of the trypanosomal enzyme.

Southern blot analysis suggested two gene copies for the NAD^+-dependent GPD in the *T.b. rhodesiense* ViTat 1.1 clone. This low gene copy number suggests that this enzyme may be an ideal candidate for attempts at gene knockout mutagenesis. Other glycosomal glycolytic enzymes have been found to have a similarly low gene copy number, with a single copy gene for each of phosphoglycerate kinase (glycosomal isoenzyme) [Osinga et al., 1985], glucosephosphate isomerase [Marchand et al., 1989] and triosephosphate isomerase [Swinkels et al., 1986], and two gene copies for each of fructose-bisphosphate aldolase [Clayton, 1985] and glyceraldehyde 3-phosphate dehydrogenase (glycosomal isoenzyme) [Michels et al., 1986]. These low gene copy numbers, when viewed in the context of the large numbers of glycosomes per trypanosome (240) [Opperdoes, 1987] and the relative contribution to total cellular protein content (4.3-8.0%) [Opperdoes, 1984; Misset et al., 1986], suggests the genes coding for these enzymes may be under the control of highly active promoters. Northern blot analysis detected a single NAD^+-dependent GPD mRNA transcript of approximately the right size (1,620 bases) to encode the 353 amino acid protein, suggesting that no major post-transcriptional modifications occur that alter the size of the transcript prior to translation.

Current studies are focusing on knockout mutagenesis of the NAD^+-dependent GPD in a fly-transmissible trypanosome strain in order to determine the effect on parasite survival within the mammalian host and the tsetse fly vector. A bacteriophage P1 library representative of the trypanosome genome has been probed with the *T.b. rhodesiense* ViTat 1.1 PCF gene, and 5 positive clones were selected. Sequencing of appropriate restriction fragments of DNA inserts in these clones is currently in progress in an attempt to obtain genomic sequences flanking the GPD genes to facilitate such mutagenesis. Results presented in this chapter suggest that knockout of the trypanosome NAD^+-dependent GPD should be feasible in the PCF life cycle stage, where this enzymatic activity is not necessary for parasite survival and thus stable mutants may be generated. However, after fly passage the BSF that differentiate in the mammalian host should die, thus reaffirming that NAD^+-dependent GPD is essential for NAD^+-regeneration in BSF trypanosomes.

MUCH OF THE WORK OUTLINED IN THIS CHAPTER HAS BEEN PUBLISHED:

Caroline E. Stebeck, Ute Frevert, Tom P. Mommsen, Erik Vassella, Isabel Roditi and Terry W. Pearson. 1996. Molecular characterization of glycosomal NAD^+-dependent glycerol 3-phosphate dehydrogenase from *Trypanosoma brucei rhodesiense*. Molecular and Biochemical Parasitology. 76: 145-158.

CHAPTER 2: Kinetoplastid Membrane Protein-11: Identification in a Variety of Kinetoplastid Parasites and Molecular Characterization in *Trypanosoma brucei* spp.

INTRODUCTION

The procyclic trypanosome major cell membrane glycoproteins collectively known as the procyclins [Richardson et al., 1988; Roditi et al., 1987; Beecroft et al., 1993; Stebeck and Pearson, 1994] effectively establish a surface coat similar to the VSG of bloodstream forms [Roditi and Pearson, 1990]. The immunodominance of VSG and the procyclins, together with their abundance and the inherent shielding effect they have on underlying membrane proteins has hampered identification of other trypanosome membrane molecules. Despite these difficulties, several trypanosome membrane molecules have been conclusively identified on the cell surface (described in detail in the general introduction), including a transferrin receptor [Schell et al., 1991;], a low density lipoprotein receptor [Coppens et al., 1988; Lee et al., 1990; Coppens et al., 1991], adenylate cyclases [Alexandre et al., 1990; Paindavoine et al., 1992], glucose transporters [Parsons and Nielsen, 1990; Seyfang and Duszenko, 1991; Bringaud and Baltz, 1992; Bringaud and Baltz, 1993; Seyfang and Duszenko, 1993] and a variety of invariant surface glycoproteins of unknown function (PSSA-2, ISGs 65 and 75) [Jackson et al., 1993a; Jackson et al., 1993b; Ziegelbauer and Overath, 1992; Ziegelbauer et al., 1992; Ziegelbauer et al., 1995].

The cell membrane of the related kinetoplastid *Leishmania* has also been partially characterized with respect to surface membrane molecules. Three different sets of membrane molecules have been extensively studied: glycoprotein 63 (gp63, the major surface proteases), lipophosphoglycans (LPG) and glycosylinositolphospholipids (GIPLs). Recently another *Leishmania* surface membrane molecule, the gene B protein, has been moderately well characterized [Coulson and Smith, 1990; Flinn and Smith, 1992; Flinn et al., 1994; Pimenta et al., 1994]. This molecule is an 18 kDa hydrophilic protein of about 1×10^5 molecules per cell that has been shown in co-capping experiments to associate with LPG on live promastigotes [Pimenta et al., 1994]. Analysis of the amino acid sequence of the gene B protein reveals it lacks an apparent signal peptide, membrane-spanning amino acid domain or consensus attachment sequence for a glycosylphosphatidylinostiol (GPI) anchor. The amino acid sequence of this protein has, however, been shown to contain a proline-rich domain composed of 5.5 repeats of a 14

amino acid motif that exhibits significant sequence similarity to the peptidoglycan-binding domain of *Staphylococcus aureus* protein A [Flinn et al., 1994]. It is through this repetitive motif that the gene B protein is proposed to associate with LPG [Pimenta et al., 1994].

Another *Leishmania* surface membrane molecule that has recently been characterized also exhibits a predilection for associating with LPG [Jardim et al., 1991; Tolson et al., 1994a; Jardim et al., 1995a; Jardim et al., 1995b]. This 11 kDa molecule was originally designated lipophosphoglycan-associated protein (LPGAP) [Jardim et al., 1991] but has since been re-named kinetoplastid membrane protein-11 (KMP-11) since it is found in a wide variety of kinetoplastid parasites [Tolson et al., 1994b]. The *Leishmania* molecule has been demonstrated to co-isolate with LPG, and evidence supporting a KMP-11-lipid bilayer interaction has been provided by carboxyfluorescein release from liposomes mediated by *L. donovani* KMP-11 [Jardim et al., 1995b]. The *Leishmania* KMP-11 shares certain characteristics with the gene B protein, including its surprising association with the surface membrane despite its hydrophilic nature and lack of apparent signal peptide, membrane-spanning amino acid domain or GPI anchor consensus attachment sequence [Jardim et al., 1995b]. Secondary structure analysis of the *Leishmania* KMP-11 predicted this molecule to have a helix-turn-helix structure and an amphipathic nature which could explain its association with cell membranes and its co-isolation with LPG [Jardim et al., 1991; Jardim et al., 1995a; Jardim et al., 1995b].

Monoclonal antibodies (mAbs) derived against the *L. donovani* KMP-11 molecule were used in immunofluorescence experiments and revealed immunological cross-reactivity with procyclic African trypanosomes, thus suggesting that these related kinetoplastids possess molecules containing cross-reactive epitopes [Tolson et al., 1989; Tolson et al., 1994b]. This chapter describes experiments that show the widespread distribution of an immunologically cross-reactive 11 kDa protein throughout various species and subspecies of African trypanosomes, in various life cycle stages and in a variety of other kinetoplastid parasites. This chapter also describes the membrane disposition and relative abundance of this molecule in *T. brucei* spp., the determination of the primary sequence of the trypanosome KMP-11 and its comparison with the *L. donovani* KMP-11. Southern blot analysis revealed that only one gene copy for the trypanosome KMP-11 exists in the *T.b. rhodesiense* ViTat 1.1 clone. This indicates that if a single KMP-11 gene copy exists in a tsetse fly-transmissible clone of African

trypanosomes, it is an excellent candidate for knockout mutagenesis and possible elucidation of KMP-11 function.

MATERIALS AND METHODS

Parasites. Bloodstream forms (BSF) of *T.b. brucei* 427 [Cross and Manning, 1973], *T.b. gambiense* TH-1 [Gray, 1972], and *T. simiae* CP-11 [Zweygarth and Rötcher, 1987] were obtained from Dr. R. Brun (Swiss Tropical Institute, Basel, Switzerland), Dr. J. Doyle (International Laboratory for Research on Animal Diseases (ILRAD), Narirobi, Kenya) and Dr. E. Zweygarth (Veterinary Laboratories, Kabete, Kenya), respectively. BSF of *T.b. rhodesiense* ViTat 1.1 and *T. congolense* IL3000 were obtained as previously described (Chapter 1, Materials and Methods). BSF parasites were harvested and purified using methods outlined in Chapter 1, with the exception of *T. simiae* CP11 BSF which were obtained by preparing buffy coats from the heparinized blood of an infected pig. Procyclic culture forms (PCF) and *T. congolense* IL3000 epimastigotes and metacyclics were established from their respective cloned bloodstream populations and were maintained in culture as previously described in Chapter 1. *L. donovani* LD3 promastigotes, *T. cruzi* Peru strain epimastigotes, *T. cruzi* Y strain trypomastigotes and *Crithidia fasciculata* strain ATCC11745 were obtained and grown as outlined in Chapter 1. *Leptomonas collosma* ATCC30261 was obtained from the American Type Culture Collection and was grown in standard Brain Heart Infusion medium (DIFCO). *Phytomonas* spp. parasites were obtained from Dr. W. Cosgrove (Minneapolis, MN, USA) and were grown in Grace's medium containing 10% heat-inactivated FBS. *Trichomonas vaginalis* strain CD-C85 (ATCC 50143) was grown as previously described [Singh et al., 1994]. *Giardia lamblia* ATCC WB strain and *Entamoeba histolytica* ATCC HM-1 IMSS were obtained from Dr. C. Chadee (Institute for Parasitology, McGill University, Montreal, Québec, Canada). The marine chrysophytes, *Isocrysis* and *Rhodomonas*, were obtained from Dr. Lou Hobson (Biology Dept. UVIC, Victoria, BC, Canada).

Transformation to procyclic culture forms. BSF of *T. congolense* IL3000 were harvested aseptically from rat blood, adjusted to 2×10^7 ml^{-1} in procyclic culture medium and 10 ml placed into each of ten 50-ml tissue culture flasks. The flasks were incubated at 26°C and at each of ten intervals parasites were harvested from one flask and cell lysates prepared as described below for subsequent gel electrophoresis and immunoblotting experiments.

Monoclonal antibodies and antisera. The mAbs L98 and L157 were derived against *L. donovani* KMP-11 (formerly called the lipophosphoglycan-associated protein) and have been described in detail elsewhere [Tolson et al., 1989; McNeely et al., 1990; Jardim et

al., 1995a; Jardim et al., 1995b]. The mAbs were originally thought to bind to an epitope within the LPG core [Tolson et al., 1989; McNeely et al., 1990], but were subsequently shown to be specific for distinct epitopes of the *Leishmania donovani* KMP-11 protein [Jardim et al., 1995a; Jardim et al., 1995b]. The anti-*T. congolense* mAb 491 [Beecroft et al., 1993] was used to measure expression of the glutamic acid/alanine-rich protein (GARP) [Beecroft et al., 1993; Bayne et al., 1993] during transformation of BSF to PCF. GARP is thought to be an analog of the *T. brucei* procyclin or procyclic acidic repetitive protein (PARP) molecules. The anti-*T. brucei* procyclin mAb TBRP1/247 [Richardson et al., 1986; Richardson et al., 1988] was used to detect PARP in fractions eluted from the octyl-Sepharose columns and in immunofluorescence studies. The mAb TH1 (anti-human transferrin, T. W. Pearson and R. P. Beecroft, unpublished) and mouse polyclonal antiserum to the *T.b. rhodesiense* ViTat 1.1 PCF NAD^+-dependent glycerol 3-phosphate dehydrogenase (Chapter 1, Discussion) were used as controls in the Triton X-114 experiments described below.

Immunofluorescence. Immunofluorescence was performed on acetone-treated *L. donovani* LD3 promastigotes, *T. b. rhodesiense* ViTat 1.1 PCF and *T. congolense* IL3000 PCF as previously described (Chapter 1, Materials and Methods). The first antibody consisted of a 1:1000 dilution of ascites fluid containing either an L98/L157 mAb mixture (each at a 1:2000 dilution) or the anti-*T. brucei* procyclin mAb (TBRP1/247, control antibody). Immunofluorescence was observed using a Zeiss standard binocular microscope fitted with an epifluorescence attachment and a Zeiss NeoFluor 63/1.25 oil immersion objective or with an inverted confocal laser scanning fluorescence microscope (Leica CLSM-Fluovert, Leica Lasertechnic, Heidelberg, Germany) fitted with a 40 x oil immersion objective.

Polyacrylamide gel electrophoresis and electrophoretic blotting. Separation of proteins in lysates of parasites by SDS-PAGE using 15% polyacrylamide gels and blotting of separated proteins onto Immobilon-P™ membranes (Millipore, Bedford, MA, USA) were performed as previously described (Chapter 1, Materials and Methods). Parasites were usually prepared for SDS-PAGE by lysis of 3 x 10^6 parasites in 7.5 µl 0.1% trifluoroacetic acid (TFA) prior to addition of an equal volume of 2x Laemmli sample buffer [Laemmli, 1970] and boiling for 2 min prior to addition of a few crystals of Tris base to neutralize the pH of the solutions. The samples were then boiled for 3 min prior to loading on the gel. This solubilization procedure eliminates the aggregation of the KMP-11 monomers which otherwise run as multiple molecular mass forms on SDS-

PAGE gels under reducing conditions. In some experiments, 3 x 10^7 parasites were lysed in the same volume. Peptides generated by endoproteinase Lys-C digestion of KMP-11 (described later) were separated using a 12-17% peptide gradient gel [Fling and Gregerson, 1986] and electroblotted onto Immobilon-P™ (Millipore Corp., Bedford, MA, USA). Either Rainbow™ coloured protein molecular mass markers or Rainbow™ coloured peptide molecular mass markers (Amersham, Oakville, ON, Canada) were run on each gel and served as markers on the Immobilon-P™ blots. In some cases gels were stained with Coomassie blue R-250, silver [Merrill et al., 1984] or Stains-all™ [Green et al., 1973]. After blotting, protein or peptide bands were detected by staining the Immobilon-P™ membranes with Coomassie blue R-250 [Matsudaira, 1987]. Immunoblotting was performed using a 1:4000 dilution of horseradish peroxidase-labeled goat anti-mouse IgG/IgM antibody (Caltag, San Fransisco, CA, USA) to detect bound primary antibody. For detection of biotin-labeled proteins, a 1:400 dilution of streptavidin-biotinylated horseradish peroxidase complex (Amersham, Oakville, ON, Canada) was used. Enhanced luminol chemiluminescence reagent (Renaissance™, Dupont NEN, Boston, MA, USA) was used as the substrate for both enzyme conjugates according to the instructions of the manufacturer. Ascites fluids containing mAbs L98 or L157, a mixture of the mAbs L98 and L157 (all at a 1:3000 dilution), mAb 491 (anti-*T. congolense* GARP) and mAb TBRP1/247 (anti-*T. brucei* procyclin) were used as first antibodies at dilutions ranging from 1:3000-1:5000.

Glycoprotein detection. After SDS-PAGE and electrophoretic blotting, carbohydrate groups attached to proteins were identified by detection of biotin incorporated into carbohydrate that had been oxidized by periodate treatment. The enhanced chemiluminescence glycoprotein detection module of Amersham (Little Chalfont, UK) employing biotin hydrazide was used according to the methods outlined by the manufacturer. Thirty micrograms of the purified 11-kDa molecule from *T.b. brucei* 427.01 PCF were loaded onto the SDS-PAGE gels. Five micrograms of the positive control, human transferrin, were also run on SDS-PAGE gels.

Biotin labeling and avidin affinity chromatography. T.b. rhodesiense ViTat 1.1 PCF (2 x 10^{10}) were labeled with NHS-SS-biotin (Pierce, Rockford, IL, USA) as outlined in Chapter 1. The resulting eluate from the avidin-agarose column was dialyzed against water, lyophilized and solubilized in 400 µl Laemmli sample buffer containing 0.1% TFA prior to SDS-PAGE and immunoblotting.

Biotin labeling and detection of biotinylated proteins. T.b. rhodesiense ViTat 1.1 PCF were surface-labeled with sulfo-NHS-biotin (Pierce, Rockford, IL, USA) using a modification of the procedure originally described by Hurley et al. [1985]. Parasites (1 x 10^9) were washed once by centrifugation (900 x g for 10 min, 4°C) with PBS (pH 7.4)/1% glucose and resuspended to 2 x 10^7 ml^{-1} in the same buffer containing 0.5 mg sulfo-NHS-biotin. Parasites were labeled for 10 min on ice with repeated microscopic monitoring of parasite viability, washed twice by centrifugation (600 x g for 10 min, 4°C) with PBS/1% glucose/10 mM lysine and the resulting pellet was resuspended in 300 µl of Laemmli sample buffer containing 0.1% TFA prior to SDS-PAGE, electrophoretic transfer to Immobilon-P™ and biotin-label detection.

Triton X-114 detergent solubilization and dot blotting. Pelleted *T.b. rhodesiense* ViTat 1.1 PCF (8 x 10^8) were solubilized with 1.2 ml 10 mM Tris-HCl (pH 7.4)/150 mM NaCl/1.0% Triton X-114 at 0°C, and phase separation was performed as described by Bordier [1981]. Briefly, the above solution was overlayed on a 300 µl cushion consisting of 6% (w/v) sucrose, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.06% Triton X-114. Following a 3 min incubation at 30°C, the mixture was centrifuged (300 x g, 3 min, 30°C) and the aqueous and detergent phases separated. The aqueous phase received 0.5% fresh Triton X-114 and the separation was repeated using the same sucrose cushion described above. The aqueous phase was subjected to a final rinse with 2% Triton X-114 without an added sucrose cushion and the detergent phase of this final separation was discarded. The aqueous and detergent phases were precipitated with 20 volumes of acetone and the concentrated pellet was resuspended in 2.0 ml distilled water. Sequential doubling dilutions of this sample were prepared and 100 µl amounts of these dilutions were dried as dots onto strips of Immobilon-P™ membranes (Millipore Corp., Bedford, MA, USA) prior to immunodetection.

Preparation of plasma membranes. T.b. rhodesiense ViTat 1.1 PCF plasma membranes were prepared from 1 x 10^{11} parasites using the procedure outlined in Chapter 1. The sample containing the plasma membrane fraction was concentrated with 20 volumes of acetone, chilled overnight at -70°C, washed twice with 20 volumes of ice-cold acetone, dispensed into six tubes and dried using a Speed-Vac concentrator (Model SVC-100H, Savant Instruments Inc, Hicksville, NY, USA). The contents of one tube were dissolved in 1.0 ml Laemmli buffer containing 0.1% TFA prior to SDS-PAGE and immunoblot analysis.

Octyl-Sepharose chromatography. Purification of PARP and the 11 kDa protein from African trypanosomes was attempted by octyl-Sepharose reverse phase chromatography using a procedure modified from those described by Ferguson et al. [1993] and by Jardim et al. [1991]. Cell pellets of *T.b. rhodesiense* ViTat 1.1 PCF (2×10^{10} cells) were extracted twice with 8 ml of chloroform/methanol/water (1:2:0.8 (v/v)). The delipidated residue was then extracted twice by sonication (5 x 15 s bursts, W-385 sonicator, Heat-Systems Ultrasonics Inc., Farmingdale, NY, USA) with 5 ml of 9% (v/v) butanol in water and centrifugation (4000 x g for 15 min, 20°C) to remove insoluble material. Supernatants were pooled, concentrated by lyophilization and dissolved in 1 ml of 40 mM NH_4OH/1 mM EDTA. This material was either applied to a 1 x 10 cm octyl-Sepharose column and eluted with 20 ml of 0.1 M ammonium acetate/5% (v/v) 2-propanol (buffer A; 4 ml h^{-1}) followed by a linear gradient to 60% (v/v) 2-propanol in water over 100 ml (12 ml h^{-1}) or was applied to a 1 x 30 cm column and eluted with 0.1 M ammonium acetate/10% 2-propanol and progressing to 70% propanol over 12 h at a flow rate of 0.3 ml min^{-1}. Column effluent was monitored at 280 nm (0.02 AUF) and 1 ml fractions were collected. Aliquots of each fraction (typically 50 µl) were dried onto wells of microplates for subsequent analysis by ELISA using mAbs L98/L157 and TBRP1/247. Immunologically positive fractions were pooled, the propanol contained in these fractions was evaporated off using an Eyela Vapor mix S-10 evaporator (Tokyo Rikakikiai Co., Tokyo, Japan) and the proteins were concentrated by lyophilization. The proteins were then analyzed by SDS-PAGE and staining/immunoblotting.

In preparation for endoproteinase Lys-C digestion, the 11 kDa protein was purified from 6×10^{10} *T.b. rhodesiense* ViTat 1.1 PCF by octyl-Sepharose chromatography as described above with slight modifications. Briefly, a 1 x 20 cm octyl-Sepharose column and an elution gradient profile beginning with 0.1 M ammonium acetate/10% 2-propanol and progressing to 70% propanol over 14.3 hours at a flow rate of 0.3 ml min^{-1} were used. Column effluent was monitored at 280 nm (0.02 AUF) and 2.5 ml fractions were collected. Twenty microlitre aliquots of each fraction were dried onto wells of microplates for subsequent ELISA analysis using a mixture of mAbs L98 and L157. Immunologically positive fractions were pooled, the propanol contained in the fractions was evaporated off as above and the proteins were concentrated by lyophilization. The purity of the 11-kDa molecule was assessed by SDS-PAGE and Coomassie blue staining.

Endoproteinase Lys-C digestion of the 11 kDa molecule. The lyophilized purified 11-kDa protein was resuspended in 50 µl of Lys-C buffer (150 mM ammonium acetate pH 8.0,

0.1% SDS) and digested with 0.02 units of Lys-C (Boehringer Mannheim Canada, Laval, Quebec, Canada) for 8 hours at 37°C. The reaction was concentrated to 25 µl in a Speed-Vac concentrator (Model SVC-100H, Savant Instruments Inc, Hicksville, NY, USA) and an equal volume of 2x Laemmli sample buffer [Laemmli, 1970] was added prior to boiling and subsequent peptide separation by SDS-PAGE.

Enzyme-linked immunosorbent assay. Aliquots (20-100 µl) of fractions from the octyl-Sepharose columns were tested by indirect ELISA as described in Chapter 1 using appropriate dilutions of ascites fluids containing the anti-*T. brucei* procyclin mAb TBRP1/247, the anti-*T. congolense* procyclin mAb 491 or a 1:1 mixture of mAbs L98 and L157.

Amino-acid microanalysis and protein microsequencing. Purified protein was pooled from the octyl-Sepharose column, lyophilized, weighed and hydrolyzed for 45 min in 6 M HCl under Argon gas. Coomassie blue-stained 11-kDa bands from Immobilon-P™ membrane were air dried and hydrolyzed similarly. Amino acid microanalysis was performed by Sandy Kielland using an Applied Biosystems model 420 derivatizer-analyzer in the University of Victoria Tripartite Microanalytical Center. Coomassie blue-stained bands of the 11-kDa protein Lys-C digest from Immobilon-P™ blots were placed directly into a gas-phase sequencer (model 470A, Applied Biosystems, Foster City, CA, USA) and sequence analysis was performed in the University of Victoria Tripartite Microanalytical Center. Sequences obtained were searched against the SWISS-PROT protein database [Altschul et al., 1990].

Agarose gel electrophoresis. Agarose gels were prepared and electrophoresed as outlined in Chapter 1.

Cloning and sequencing. The *E. coli* strains XL-1 Blue (tetracycline^R; Stratagene, La Jolla, CA, USA) and DH5α (Gibco, BRL, Burlington, ON, Canada) were used in transformation experiments according to the method of Hanahan et al. [1983] and standard gene cloning techniques were applied as described by Sambrook et al. [1989]. Bacterial cultures were routinely grown in either LB medium or 2 x YT medium [Sambrook et al., 1989]. The λgt22A PCR fragment (see below) was cloned into the Bluescript™ SK$^+$ vector (Stratagene, La Jolla, CA, USA). The *Eco*RI fragment of the positive cosmid clone (see below) was cloned into either the Bluescript™ SK$^+$ vector (Stratagene) or the Litmus™ 39 vector (New England Biolabs, Beverly, MA, USA) to

allow sequencing in both directions. The amplified cDNA PCR fragment (see below) and the genomic PCR fragment amplified using primers 1 and 3 (see below) were cloned into either the pGEM-T™ vector (Promega, Madison, WI, USA) or the Litmus™ 39 vector, also to allow sequencing in both directions. In all cases the DNA fragments to be cloned were recovered from low-melting-temperature agarose using Wizard™ PCR Preps purification system. Double-stranded DNA was prepared using Nucleobond™ AX cartridges (Macherey-Nagel, Düren, Germany). Single-stranded DNA was prepared using a modification of the protocol described by Vieira and Messing [1987] (described in detail in Chapter 1). Sequencing of both double-stranded and single-stranded DNA was performed using Sequenase™ Version 2.0 DNA sequencing kits (United States Biochemical, Cleveland, OH, USA). Both universal sequencing primers and internal primers designed from DNA sequences were used. The 5' pGEX Sequencing Primer (Pharmacia, Piscataway, NJ, USA) was used for sequencing of the pGEX-2T overexpression construct (see below).

cDNA expression library screening. A cDNA expression library was kindly supplied by Dr. Isabel Roditi (University of Bern, Bern, Switzerland). The unidirectional library was constructed using the Superscript™ Lambda System (Gibco BRL, Paisley, Scotland) according to the manufacturer's instructions. Briefly, *T.b. brucei* GARP 16 poly $(A)^+$ RNA [Hehl et al., 1995] was reversed transcribed, second strand cDNA synthesis was performed, *Not*I and *Sal*I adapters were added and the double stranded cDNA was directionally cloned into the λgt22A cloning vector [Han and Rutter, 1987]. The library host strain *E. coli* Y1090 (Gibco BRL, Burlington, ON, Canada) was propagated in LB medium supplemented with 0.2% maltose and 0.1% $MgSO_4$. Five plates of the cDNA library were prepared by adding the λgt22A library (10^5 pfu/plate) to 100μl of *E. coli* Y1090 overnight culture, incubating 20 min at 37°C, adding 8 ml molten (48°C) H top agarose (1% bacto-tryptone, 86 mM NaCl, 0.75% agarose (w/v)) and pouring onto an H agar plate (1% bacto-tryptone, 86 mM NaCl, 1.5% bacto-agar). The plates were incubated 3.5 to 5 hours at 42°C, overlayed with nitrocellulose filters (150 mm; Schleicher and Schuell, Keene, NH, USA) impregnated with 100mM isopropylthio-β-D-galactoside (IPTG) (Stratagene, La Jolla, CA, USA) and incubated at 37°C overnight. Duplicate lifts were prepared by removing the filters and overlaying the plates with fresh IPTG-impregnated filters prior to a second overnight incubation. Filters were stored in PBS at 4°C until the immunoscreening step. Filters were screened with the L98/L157 mAb mixture according to Amersham's tech tip #133 (R932148 for use with the enhanced chemiluminescence (ECL)™ system; Amersham, Oakville, ON, Canada). Briefly, filters

were blocked in PBS/10% milk powder for 10 minutes at room temperature, washed with 200 ml PBST (3 x 30 sec; PBS/0.1% (v/v) Tween-20) and incubated with the L98/L157 mAb mixture (1:1000 dilution of each mAb in PBS) for 30 minutes at room temperature. Filters were washed 2 x 10 minutes with PBST, incubated for 30 minutes at room temperature with goat anti-mouse IgG/IgM horseradish peroxidase-conjugate (Caltag, San Francisco, CA, USA) diluted 1:5000 in PBS and subjected to 2 final washes with PBST for 10 minutes each. The ECL™ reagent (Renaissance™, Dupont NEN, Boston, MA, USA) was used as the substrate for horseradish peroxidase according to the instructions of the manufacturer. Any plaques exhibiting immunoreactivity were picked from the original plates using a sterile Pasteur pipette, placed into 1 ml of SM buffer (1 M Tris-HCl pH 7.5, 2% gelatin, 8 mM $MgSO_4$, 100 mM NaCl), and incubated overnight at 4°C. Secondary screening to verify positivity was performed as outlined above using the phage suspension diluted 1:100 in SM. Well isolated plaques remaining positive upon secondary screening were placed into 1 ml SM buffer containing 0.3% chloroform and stored at 4°C.

Preparation of high titre phage stock. A high titre λgt22A phage stock was prepared using the method of O'Toole and Foster [1988]. Briefly, 100 µl of an overnight *E. coli* Y1090 culture was dispensed into each of four tubes and subsequently inoculated with either 1, 2, 3 or 4 well isolated plaques selected from a fresh overnight plate of *E. coli* Y1090 infected with positive phage particles. The suspensions were mixed on a rotary mixer (Super-mixer, cat. no. 1290, Lab-line instruments Inc., Melrose Park, IL, USA), incubated at 37°C for 20 min, 2.5 ml of molten (48°C) H top agarose was added and the mixture was poured onto an H agar plate. Following incubation at 37°C for 4 hours the plates were monitored every 30 minutes for the occurrence of confluent lysis. The plate exhibiting confluent lysis by 6 hours was selected and phage harvested from this plate by the addition of 3 ml SM and subsequent removal of the top agarose by scraping into a 15 ml tube (Sarstedt, Newton, NC, USA). One hundred microlitres of chloroform were added and the mixture was mixed on a rotary mixer to release the phage particles. The tube was then centrifuged (4000 x g, 10 min, 4°C) and the resulting supernatant used as the high titre phage stock. The titre of the phage stock was determined using established methods [Sambrook et al., 1989].

Preparation of phage.[8] Phage particles were prepared using the methods of O'Toole and Foster [1988] with minor modifications. Briefly, four flasks containing 300 ml of complete medium (1% casamino acids, 1x M9 salts, 2% glucose, 2% maltose, 5mM $MgCl_2$ and 1mM $CaCl_2$) [Sambrook et al., 1989] were each inoculated with 0.5 ml of an overnight *E. coli* Y1090 culture and 100 µl of the high titre phage stock from above (10^9 pfu). The flasks were incubated at 37°C until lysis of the bacteria occurred (5-9 hours), at which time 5 ml of chloroform was added to each flask and the suspension was mixed well and incubated 15 minutes at room temperature. Insoluble material was removed by centrifugation (10,000 x g, 10 min, 4°C) and the supernatant decanted into a fresh flask. Sodium chloride and polyethylene glycol-8000 were added to final concentrations of 0.5 M and 10% (w/v), respectively, and the suspension was mixed well. Following overnight incubation at 4°C the phage particles were pelleted by centrifugation (4000 x g, 30 min, 4°C), the supernatant removed by decanting and the pellet re-centrifuged. The resulting phage pellet was resuspended in 7.5 ml of 10 mM Tris-HCl pH 7.5, 0.25 M NaCl and 5 mM $MgCl_2$ and layered onto a cesium chloride (CsCl) block gradient (1.7, 1.55 and 1.3 mg ml^{-1} CsCl) in a Beckman polyallomer centrifuge tube (14 x 89 mm, Beckman Instruments, Palo Alto, CA, USA). Following centrifugation (77,000 x g, 90 min, 4°C) the purified phage particles were recovered between the 1.55 and 1.3 mg ml^{-1} CsCl density layers and stored at 4°C.

Isolation of phage DNA. The phage DNA was released from the phage capsids using the method of O'Toole and Foster [1988]. Briefly, 0.1 volumes of 0.2 M Na_2-EDTA, 2M Tris-HCl pH 8.5 and an equal volume of formamide were added to the CsCl purified phage particle preparation and the suspension was mixed on a vortex mixer. Following overnight incubation at room temperature the purified phage DNA was ethanol precipitated using conventional methods [Sambrook et al., 1989] and the precipitate resuspended in 40 µl TE buffer (10 mM Tris-HCl pH 7.4, 1 mM Na_2-EDTA).

PCR amplification of the cDNA insert. The cDNA insert from the λgt22A positive phage particle was isolated using polymerase chain reaction (PCR [Saiki et al., 1988]) amplification techniques. The PCR reaction mixture (100 µl total volume) consisted of 0.25 µM Lambda gt11 primer (forward), 0.25 µM Lambda gt11 primer (reverse) (both primers from New England Biolabs, Beverly, MA, USA), 1 x Taq DNA polymerase buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 2 mM each dATP,

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[8] This work was performed by Corinna Tuckey, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

dCTP, dGTP, dTTP (Pharmacia, Piscataway, NJ, USA), 10 µl purified phage DNA (see above) and 2.5 units *Taq* DNA polymerase (Pharmacia, Piscataway, NJ, USA). The following PCR protocol was performed in a Thermolyne Temp•Tronic™ Thermal Cycler (Barnstead/Thermolyne, Dubuque, IA, USA): 50°C annealing, 74°C extension, 30 cycles. "Hot start" PCR [Chou et al., 1992] was performed for this and all subsequent PCR amplifications as outlined in Chapter 1, Materials and Methods. Following PCR the amplification product was digested with *Not*I and *Sal*I (New England Biolabs, Beverly, MA, USA) and ligated to a similarly digested Bluescript™ SK^+ vector.

Overexpression studies. To express the 92 residue-trypanosome 11 kDa molecule as a recombinant fusion protein, forward and reverse PCR primers were designed from the nucleotide sequence and used to amplify the coding region of the 11 kDa molecule from *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA in preparation for cloning into the Glutathione-S-Transferase (GST) gene fusion vector pGEX-2T (Pharmacia, Piscataway, NJ, USA). Primer 1 (forward) [5'-CGGGATCCACGAGTAAAAAGATGAGTAATG-3'] was complementary to the 5' end of the coding region and introduced a *Bam*HI site (underlined sequence) one codon downstream of the ATG start codon. Primer 2 (reverse) [5'-CCGGAATTCTACTAATAAACTAAAAACTACG-3'] was complementary to the 3' end of the noncoding region and introduced an *Eco*RI site 31 bp downstream of the natural stop codon. Primers for this and all subsequent amplifications were designed using OLIGO™ Version 4.0 software (National Biosciences, Inc., Plymouth, MN, USA). The PCR amplification reaction mixture (100 µl total volume) consisted of 0.25 µM forward primer, 0.25 mM reverse primer, 1 x *Taq* DNA polymerase buffer, 2 mM each dATP, dCTP, dGTP, dTTP (Pharmacia, Piscataway, NJ, USA), 500 ng genomic DNA and 2.5 units *Taq* DNA polymerase (Pharmacia, Piscataway, NJ, USA). Following PCR (50°C annealing, 74°C extension, 30 cycles) the amplification product was digested with *Bam*HI and *Eco*RI, ligated to a similarly digested pGEX-2T overexpression vector and transformed into *E. coli* DH5α. Prior to overexpression the nucleotide sequence spanning the pGEX-2T-trypanosome 11 kDa molecule ligation junction was subjected to double-stranded DNA sequencing to verify the sequence was in the correct reading frame for expression. Overexpression and subsequent purification of the GST-trypanosome 11 kDa fusion protein was performed as described in Chapter 1, except that the fusion protein was not subjected to thrombin cleavage for removal of the trypanosome 11 kDa molecule from the GST fusion protein. Control overexpression studies were performed using the pGEX-2T vector alone. Protein samples resulting from

the overexpression experiments were prepared for SDS-PAGE and immunoblotting analyses by the addition of an equal volume of 2x Laemmli sample buffer.

Cosmid library screening. A cosmid library was kindly supplied by Dr. David Campbell and Sandy Wong (UCLA, CA, USA). The library was prepared using genomic DNA from *T.b. brucei* 427 PCF which was cloned into the c2X75 cosmid vector [Campbell, 1989]. Briefly, the genomic DNA was partially digested with *Sau*3AI to generate inserts of approximately 35-42 kb which were subsequently cloned into the *Bam*HI site of the cosmid vector. A 242 bp hybridization probe corresponding to part of the coding region (amino acids 1 to 81) of the KMP-11 molecule from *Leishmania donovani* was prepared from a 3.4 kb *Sac*I cosmid clone fragment that has been described elsewhere [Jardim et al., 1995b]. The probe was purified from low-melting temperature agarose (Promega, Madison, WI, USA) using the Wizard™ PCR Preps purification system (Promega) and 4 µg of the prepared probe were end labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) and [γ-^{32}P]dATP (specific activity 3000 Ci/mmol; Dupont Canada Inc., Mississauga, ON, Canada) using established methods [Sambrook et al., 1989]. Briefly, 4 µg of probe, 1x PNK buffer (5 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 5 µg ml^{-1} bovine serum albumin), 50 µCi [γ-^{32}P]dATP and 20 units of T4 polynucleotide kinase were made up to a total reaction volume of 30 µl and incubated at 37°C for 2 hours. The labeled probe was purified from unincorporated label by passage through a NucTrap™ push column (Stratagene, La Jolla, CA, USA). The library was propagated in the *E. coli* host strain 490A and plate colonies transferred onto three nitrocellulose filters (150 mm; Schleicher and Schuell, Keene, NH, USA) at a density of approximately 1000 cosmid clones/filter using standard techniques [Sambrook et al., 1989]. Filters were prehybridized for 2 hours in a solution of 90 ml 6x SSC (0.9 M NaCl, 0.09 M sodium citrate), 10 ml 50x Denhardt's reagent (1 g Type 400 Ficoll, 1 g polyvinylpyrrolidone and 1 g bovine serum albumin in 100 ml of H_2O), 0.1% SDS and 0.1% sodium pyrophosphate, followed by overnight hybridization at 55°C with mild agitation in the same buffer with added probe (2 x 10^5 c.p.m./ml; specific radioactivity 5 x 10^6 c.p.m./µg). Filters were washed four times for 10 minutes with 100 ml of 3x SSC/0.1% SDS at 55°C with constant shaking and were air-dried and autoradiographed. Any cosmid clone demonstrating reactivity with the heterologous probe was rescreened under similar conditions to verify positivity. In order to decrease the insert size of the cosmid clone for sequencing, DNA was isolated from the secondary screening of the positive cosmid clone using Nucleobond™ AX cartridges (Macherey-Nagel, Düren,

Germany), digested with *Eco*RI (New England Biolabs, Beverly, MA, USA) and separated on a 1% agarose (Promega, Madison, WI, USA) gel. The DNA was transferred to Zeta-Probe® membrane (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, prehybridized 5 minutes at 65°C in 0.25 M Na_2HPO_4, 7% SDS and hybridized overnight at 65°C with mild agitation in the same buffer with the *L. donovani* KMP-11 probe described above. The membrane was washed 2 times for 60 minutes each at 65°C with 100 ml of 20 mM Na_2HPO_4 pH 7.2, 5% SDS, followed by 2 washes for 60 minutes each at 65°C with 100 ml of 20 mM Na_2HPO_4 pH 7.2, 1% SDS. The membrane was then air-dried and autoradiographed. A hybridizing *Eco*RI fragment was selected and subcloned as described above.

RNA-PCR. T.b. rhodesiense ViTat 1.1 PCF total RNA was isolated from trypanosome lysates by the procedure described in Sambrook et al. [1989], and poly(A)$^+$ RNA was subsequently purified using a Pharmacia mRNA Purification Kit (Pharmacia, Piscataway, NJ, USA). First-strand cDNA was synthesized using SUPERSCRIPT™ II RNase H$^-$ Reverse Transcriptase (Gibco BRL, Burlington, ON, Canada) from poly(A)$^+$ RNA according to the manufacturer's instructions. Briefly, the cDNA synthesis reaction mixture (20 µl total volume) consisted of 500 ng oligo $(dT)_{12-18}$, 0.4 µg poly(A)$^+$ RNA, 1x First Strand Buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 0.5 mM each dATP, dCTP, dGTP and dTTP and 200 units SUPERSCRIPT™ II. Prior to PCR amplification the cDNA was incubated with 2 units of RNase H at 37°C for 20 minutes to destroy the RNA which can interfere with PCR amplification of cDNA. The KMP-11 upstream non-coding and 5' coding regions were amplified using a 5' mini-exon (forward) primer [5'-CAGTTTCTGTACTATATTG-3'] [Borst, 1986; Walder et al., 1986] and a specific 3' internal (reverse) primer [5'-TTCATCTCAGGGGACAGCG-3'] designed from a partial trypanosome KMP-11 DNA sequence obtained from sequencing the *Eco*RI cosmid subclone. The PCR amplification reaction mixture (100 µl total reaction volume) consisted of 0.25 µM forward primer, 0.25 µM reverse primer, 1 x *Taq* DNA polymerase buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 2 mM each dATP, dCTP, dGTP and dTTP (Pharmacia, Piscataway, NJ, USA), 2.23 µl cDNA (see above) and 2.5 units *Taq* DNA polymerase (Pharmacia, Piscataway, NJ, USA). The following PCR protocol was performed: 35°C annealing, 74°C extension, 2 cycles; 40°C annealing, 74°C extension, 35 cycles.

Genomic PCR. T.b. rhodesiense ViTat 1.1 PCF genomic DNA was isolated from trypanosome lysates by the procedure described in Sambrook et al. [1989]. The KMP-11 nucleotide sequence obtained from sequencing of the *Eco*RI cosmid subclone and the KMP-11 PCR product was used to design forward and reverse PCR primers to amplify the entire coding region of the KMP-11 molecule from trypanosome genomic DNA. Primer 1 (forward) [5'-ATGGCCACCACATACGAAG -3'] was complementary to the 5' end of the coding region and encompasses the ATG start codon. Primer 2 (reverse) [5'-TCATTTTCCGGGGAACTG -3'] was complementary to the 3' end of the coding region and ends with the natural stop codon. Primer 3 (reverse) [5'-AATGGAAAGAAAATGAGAGGTG-3'] was complementary to a region 15 bp downstream of the natural stop codon and was designed from this region of DNA in order to avoid any primer-induced errors in the DNA sequence corresponding to the coding region. The PCR amplification reaction mixtures were prepared as outlined in the methods for overexpression studies (see above). Two separate PCR reactions were performed using combinations of primers 1 and 2 and primers 1 and 3. The following PCR protocols were performed for the primer 1 and 2 and primer 1 and 3 combinations, respectively: 58°C annealing, 74°C extension, 30 cycles; 57°C annealing, 74°C extension, 30 cycles.

Database searches, sequence alignment and deduced amino acid sequence characterization. Database searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm [Altschul et al., 1990] and a non-redundant protein database (includes SWISS-PROT, PIR, GenPept and GenPept updates). The trypanosome nucleotide sequences were translated and sequence alignment comparing the KMP-11 molecules of *T. brucei* and *L. donovani* was performed using GeneWorks® software (IntelliGenetics, Mountain View, CA, USA). The percentage of positional identity between sequences was calculated from the number of identical residues between aligned sequences. The molecular mass was calculated using the MacProMass™ v1.05 software (Beckman Research Institute, City of Hope, Duarte, CA, USA). The pI and net charge at physiological pH (7.4) of the translated product were calculated using the PC/Gene™ software (IntelliGenetics, Mountain View, CA, USA). KMP-11 secondary structure was predicted using the algorithm of Garnier et al. [1978] and the results were plotted on an α-helical wheel plot[9].

[9] The α-helical wheel plot was kindly prepared by Albert Labossiere, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

Southern and Northern blot analyses. Southern and Northern blots were prepared as described in Chapter 1. Twenty five nanograms of the 279 bp genomic PCR fragment corresponding to the entire KMP-11 coding region amplified using primers 1 and 2 (see above) were labeled with [α-^{32}P]dATP (specific activity 3000 Ci/mmol) using the Multiprime DNA labeling system (Amersham, Oakville, ON, Canada) according to the manufacturer's instructions. The Southern and Northern blots were prehybridized, hybridized with the labeled homologous probe (1.05×10^6 c.p.m. ml^{-1}; specific radioactivity 8.4×10^8 c.p.m. μg^{-1}) and subsequently washed and autoradiographed as described above.

Bacteriophage P1 library screening. A high density filter (SM7 No 8) containing *L. donovani* strain 2903 and *T. brucei* strain 927 bacteriophage P1 libraries (described in detail in Chapter 1) was probed with the α-^{32}P-labeled trypanosome KMP-11 genomic PCR fragment generated for the Southern and Northern blot analyses (described above). Prehybridization, hybridization, filter washing and autoradiography were performed as described in Chapter 1.

Processing of bacteriophage P1 clones.[10] P1 phage clones demonstrating reactivity with the trypanosome KMP-11 probe were selected and DNA was isolated from these clones and rescreened to verify positivity as described in Chapter 1. One positive trypanosome P1 clone was selected for further analysis. In order to decrease the insert size of the P1 clone in preparation for obtaining flanking regions of the KMP-11 gene, DNA was isolated from the positive P1 clone, digested with *SalI* (New England Biolabs, Beverly, MA, USA) and separated on a 0.6% agarose (Promega, Madison, WI, USA) gel. The DNA was transferred to Zeta-Probe® membrane (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, probed with the trypanosome KMP-11 gene as described for the Southern and Northern blot analyses and one hybridizing DNA fragment was selected. Although beyond the scope of this thesis, future work will use these genomic flanking sequences for knockout mutagenesis of the KMP-11 gene.

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[10]This work was performed in part by Michael Bridge, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

RESULTS

Detection of the 11 kDa molecule in different species of African trypanosomes.[11] Lysates of various species and subspecies of African trypanosomes were subjected to SDS-PAGE and the separated proteins were tested in immunoblots with a mixture of the *L. donovani* KMP-11-specific mAbs L98 and L157. The results are shown in Figure 17, panel A. An 11 kDa immunoreactive band was found in PCF of all three *T. brucei* subspecies (*T.b. brucei*, *T.b. rhodesiense* and *T.b. gambiense*, lanes 2-4, respectively), in both Kilifi- and savannah-type *T. congolense* (lanes 5 and 6, respectively) and in *T. simiae* (lane 7). Lane 1 shows the reactivity of the mAbs against *L. donovani* LD3, the parasites from which the leishmania KMP-11 protein was isolated for derivation of the mAbs [Tolson et al., 1989]. The *T.b. brucei* 427.01 PCF showed a fainter 11 kDa band than the other trypanosomes (lane 2), an observation that was repeated in several experiments.

Distribution of the 11 kDa molecule throughout the African trypanosome life cycle.[11] Expression of the 11 kDa molecule was measured in various life-cycle stages by immunoblotting using the L98/L157 mAb mixture (Figure 17, panel B). Lane 1 is a control lane showing immunoreactivity with the 11 kDa protein of *L. donovani* LD3 promastigotes. A strongly immunoreactive 11 kDa band was observed in *T. congolense* IL3000 PCF, epimastigotes and metacyclics (lanes 3-5, respectively). A faint immunoreactive band was observed in the DEAE-purified *T. congolense* BSF (lane 2). The low levels of the 11 kDa protein in the DEAE-purified BSF was confirmed in several experiments. The expression of the 11 kDa protein was followed by immunoblotting during transformation of *T. congolense* IL3000 from BSF to PCF (Figure 17, panel C). The 11 kDa band was present at low levels in the BSF and increased as early as 2 h after initiating transformation to PCF, increasing to maximum levels by 48-72 h.

Detection of the 11 kDa molecule in other organisms.[11] Expression of the 11 kDa antigen in a variety of other parasites was investigated by immunoblotting using the L98/L157 mAb mixture. The results are shown in Figure 18. Lanes 1 and 2 are control lanes showing immunoreactivity with the 11 kDa protein of *L. donovani* LD3 promastigotes and *T.b. rhodesiense* ViTat 1.1 PCF, respectively. An 11 kDa immunoreactive band was observed in *T. cruzi* trypomastigotes (lane 3) and epimastigotes (lane 4), although the immunoreactivity exhibited by the trypomastigotes was substantially weaker than that

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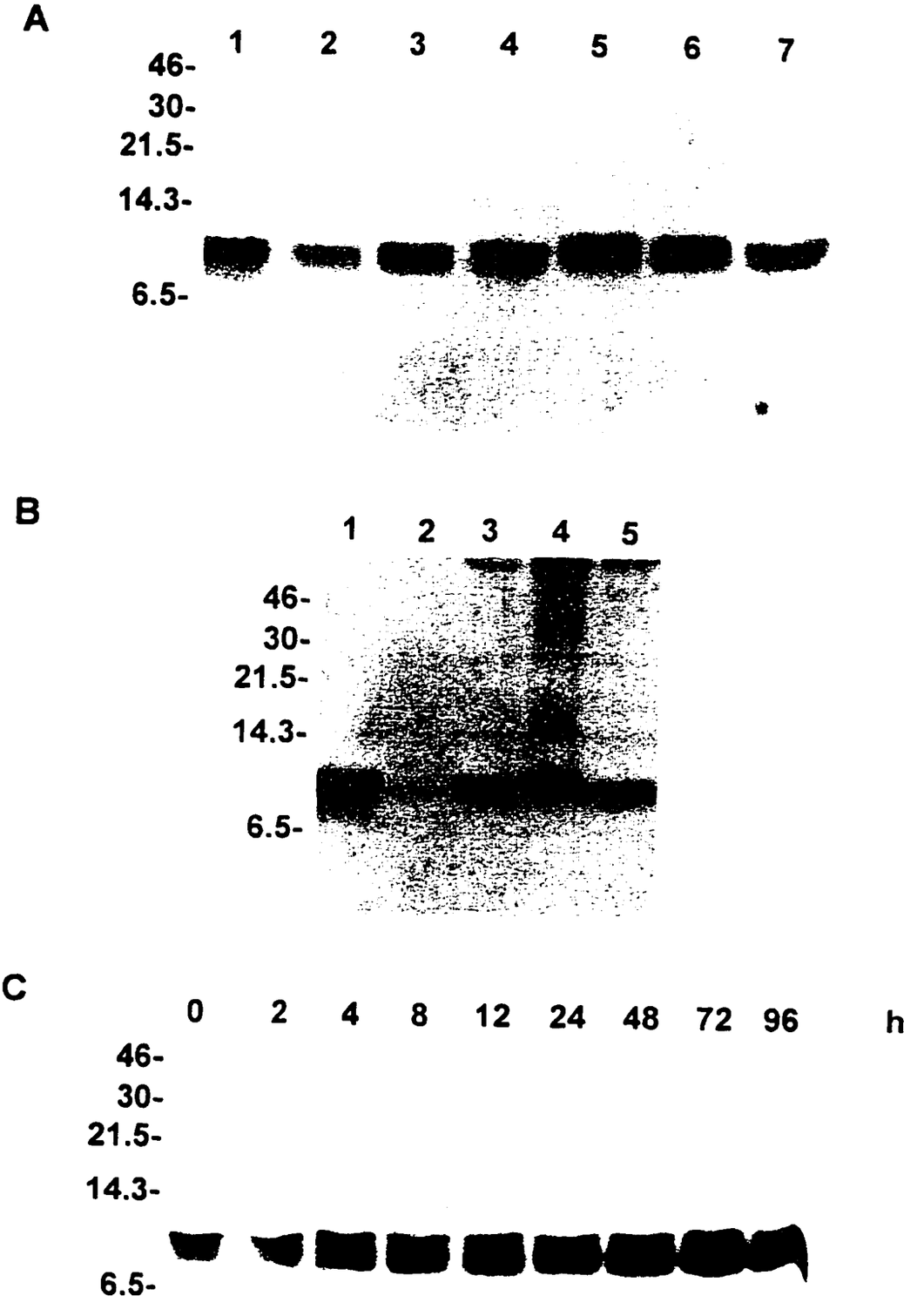
[11] This work was performed in part by Robert Beecroft, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

Figure 17 Detection of the 11 kDa molecule in various species and subspecies of African trypanosomes and throughout the trypanosome life cycle. **A.** Immunoblot analysis of SDS-PAGE separated proteins in whole cell lysates of *Leishmania donovani* and various species and subspecies of African trypanosomes. Lane 1, *L. donovani* LD3 promastigotes (positive control). Lane 2, *T.b. brucei* 427.01 PCF. Lane 3, *T.b. rhodesiense* ViTat 1.1 PCF. Lane 4, *T.b. gambiense* TH-1 PCF. Lane 5, *T. congolense* K45/1 PCF (Kilifi-type). Lane 6, *T. congolense* IL-3000 PCF (savannah-type). Lane 7, *T. simiae* CP-11 PCF. **B.** Immunoblot analysis of the 11 kDa protein expression throughout the life cycle of *T. congolense* IL-3000. Lane 1, *L. donovani* LD3 promastigotes (positive control). Lane 2, *T. congolense* BSF. Lane 3, *T. congolense* PCF. Lane 4, *T. congolense* epimastigotes. Lane 5, *T. congolense* metacyclics. **C.** Expression of the 11 kDa protein during transformation of *T. congolense* IL-3000 from BSF to PCF. Lane assignments correspond to the intervals (in hours) at which samples were taken for immunoblot analysis. Protein molecular mass markers (in kilodaltons) are indicated on the left of each panel.


A
1 2 3 4 5 6 7
46-
30-
21.5-
14.3-
6.5-
B
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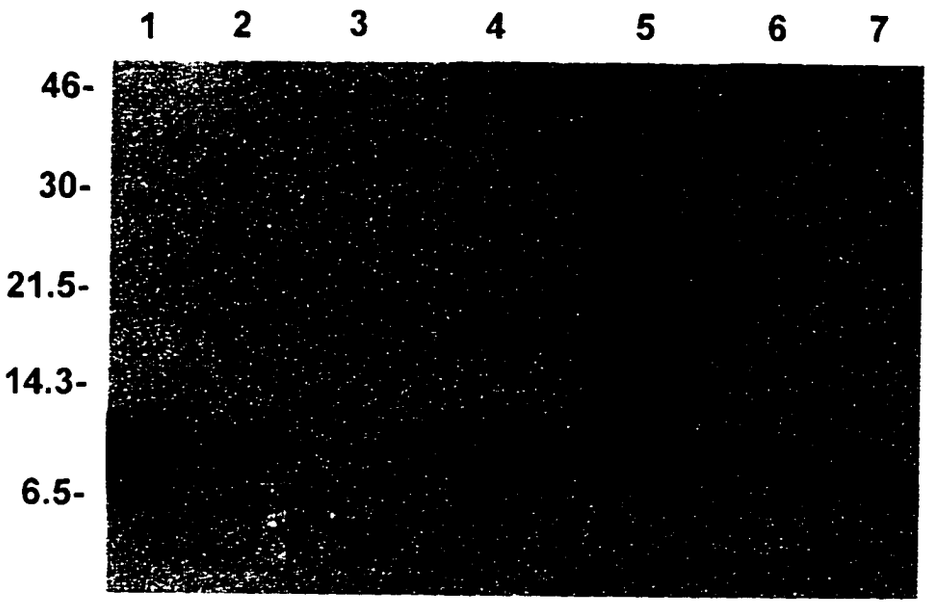
46-
30-
21.5-
14.3-
6.5-
C
0 2 4 8 12 24 48 72 96 h
46-
30-
21.5-
14.3-
6.5-

Figure 18 Immunoblot analysis of the 11 kDa protein in various species of kinetoplastid parasites. Lane 1, *L. donovani* LD3 promastigotes (positive control). Lane 2, *T.b. rhodesiense* ViTat 1.1 PCF (positive control). Lane 3, *T. cruzi* trypomastigotes. Lane 4, *T. cruzi* epimastigotes. Lane 5, *Crithidia fasciculata*. Lane 6, *Leptomonas collosma*. Lane 7, *Phytomonas* spp. Protein molecular mass markers (in kilodaltons) are indicated on the left.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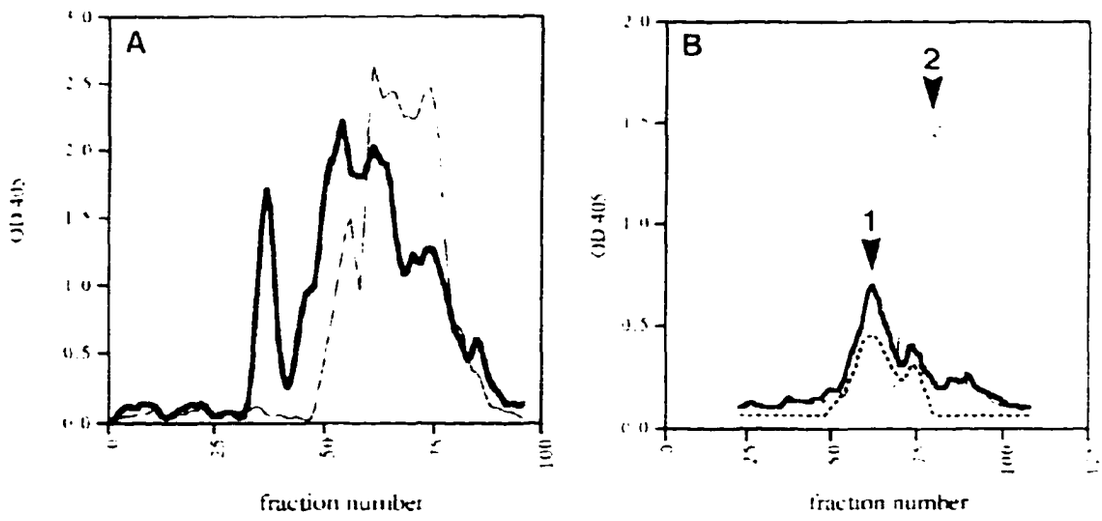
observed in the epimastigotes. This observation was repeated in several experiments. An 11 kDa immunoreactive band was present in lysates of *Crithidia fasciculata* (lane 5), *Leptomonas collosma* (lane 6) and *Phytomonas* spp. (lane 7) parasites. The abundance of the 11 kDa protein in these last three parasites appeared to be much less than in the trypanosomes and leishmania since it was necessary to load 10-fold more material onto the SDS-PAGE gels to obtain equivalent positive signals in the immunoblots. No 11 kDa band was detected in the mammalian macrophage cell line J 8114D, in the X63-Ag8.653 murine hybridoma cell line, in *Trichomonas vaginalis, Giardia lamblia, Entamoeba histolytica* or in the non-pathogenic marine chrysophytes (also belonging to the superfamily mastigophora) (blots not shown).

Purification of the 11 kDa protein from African trypanosomes.[11] The 11 kDa molecule was initially purified from trypanosomes by two sequential passages over octyl-Sepharose columns. Fractions eluted from a 1 x 10 cm octyl-Sepharose column were tested by ELISA for reactivity with both the L98/L157 mAb mixture and the anti-*T. brucei* procyclin mAb TBRP1/247. As shown in Figure 19, panel A, the 11 kDa protein and procyclin co-eluted from the octyl-Sepharose column at approximately the same concentration of 2-propanol. Fractions containing both the 11 kDa protein and procyclin were pooled and subjected to chromatography over a 1 x 30 cm octyl-Sepharose column at a slower flow rate. Effluent from this column was monitored at 280 nm and fractions were assayed for reactivity with mAbs L98/L157 and mAb TBRP1/247 (Figure 19, panel B). Clear separation of the immunoreactive peaks was achieved. Fractions containing the 11 kDa molecule and procyclin were pooled separately and subjected to SDS-PAGE followed by staining with Coomassie blue, Stains-All ™, silver, and immunoblotting. The results are shown in Figure 20. The peak containing immunoreactivity with the mAb L98/L157 mixture (panel A, lane 1) contained a dark 11 kDa band and a faint 45 kDa band. When stained with Coomassie blue the peak showed only an 11 kDa band (panel B, lane 1). Silver staining revealed the 45 kDa band as well as the darker 11 kDa band (panel B, lane 2). The peak containing anti-procyclin immunoreactivity (Figure 20, panel A, lane 2) showed only the broad diffuse band characteristic of procyclin as detected by Stains-All™ (not shown).

Characterization of the membrane disposition of the T.b. rhodesiense 11 kDa molecule. *T.b. rhodesiense* ViTat 1.1 PCF were labeled with either NHS-SS-biotin or sulfo-NHS-

[11] This work was performed in part by Robert Beecroft, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

Figure 19 Detection of KMP-11 and procyclin by ELISA of octyl-Sepharose High Performance Liquid Chromatography (HPLC)-fractionated *T.b. brucei* 427.01 PCF proteins. **A.** Fast run (500 min). **B.** Slow run (720 min). Bold line: reactivity with the L98/L157 mAb mixture. Thin line: reactivity with mAb 247 (anti-procyclin). Dotted line: A280 nm profile. The numbers 1 and 2 (fractions 50-70 and 71-90, respectively) indicate the peaks pooled for immunoblot and SDS-PAGE analysis in Figure 20.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B
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2
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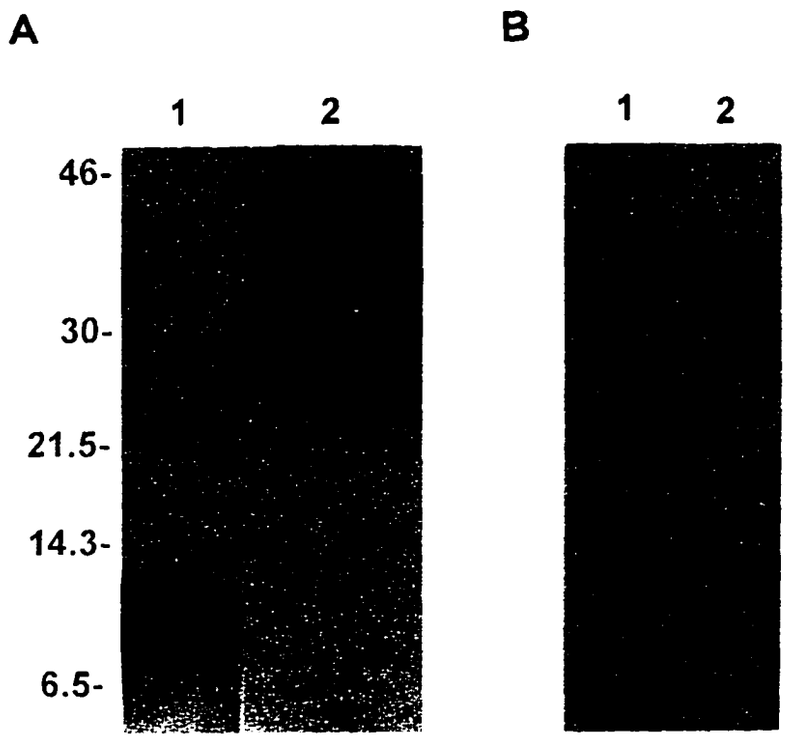
fraction number

Figure 20 Immunoblot and SDS-PAGE analyses of proteins in pooled peaks from octyl-Sepharose HPLC-separated proteins of *T.b. brucei* 427.01 PCF. **A.** Immunoblot analysis. Lane 1, peak 1/mAb L98/L157 mixture. Lane 2, peak 2/mAb 247 (anti-procyclin). **B.** SDS-PAGE analysis. Lane 1, peak 1 (Coomassie blue stained). Lane 2, peak 1 (silver stained).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biotin and subjected to avidin-affinity chromatography followed by SDS-PAGE and immunoblotting or SDS-PAGE and biotin-label detection, respectively, to examine the surface accessibility of the trypanosome 11 kDa molecule. No 11 kDa protein was detected in several experiments (results not shown). Immunofluorescence on living procyclic trypanosomes (*T. congolense* IL3000, *T.b. rhodesiense* ViTat 1.1) and on living leishmania promastigotes (*L. donovani* LD3) was negative with the L98/L157 mAb mixture (results not shown). *T.b. rhodesiense* ViTat 1.1 PCF plasma membranes were prepared and subjected to immunoblot analysis using the L98/L157 mAb mixture, and an immunoreactive band at 11 kDa was found to be present in this preparation (results not shown). Dot blotting experiments using the L98/L157 mAb mixture as a probe showed that the 11 kDa molecule was extracted almost completely in the detergent phase of Triton X-114 (Figure 21).

Characterization of the T. brucei 11 kDa molecule.[11] No carbohydrates were detected on the purified 11 kDa molecule in several experiments using the enhanced chemiluminescence-biotin hydrazide procedure whereas the positive control, human transferrin, was positive in all experiments even when only one-sixth as much protein was loaded onto the SDS-PAGE gels (data not shown). Amino acid microanalysis of the purified 11 kDa protein purified from trypanosomes was performed on quadruplicate samples from an octyl-Sepharose peak. The results are shown in Table 6. The protein shows relatively high levels of Asx and Glx but otherwise does not have a distinctive composition. Amino acid analysis was also performed on the 11 kDa band from Immobilon-P™ membrane after SDS-PAGE and blotting. Similar results were obtained (data not shown). Gravimetric determination and the yields from amino acid analysis indicated that the 11 kDa protein was expressed at approximately 2×10^5-1×10^6 molecules per cell for the *T.b. brucei* 427.01 PCF clone.

Immunofluorescence microscopy.[11] The binding of mAb L157 mAb to acetone-permeabilized parasites was determined by indirect immunofluorescence (Figure 22). Panel A shows the immunoreactivity on *L. donovani* LD3 promastigotes. The fluorescence was spread throughout much of the organism but also showed a string-like pattern along the flagellum which ended with two fluorescent spots at the flagellar base. This distinctive string-like fluorescence pattern was also observed with mAb L157 on *T. congolense* IL3000 PCF (panel B) and on both *T. brucei* and *T. congolense* BSF (results

[11] This work was performed in part by Robert Beecroft, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

Figure 21 Dot-blot analysis of *T.b. rhodesiense* ViTat 1.1 PCF 11 kDa molecule after Triton X-114 detergent solubilization. **A.** Triton X-114 detergent phase. **B.** Triton X-114 aqueous phase. Lanes in both panels A and B (left to right) are dilutions 1:2 - 1:32, respectively. **C.** Controls. Left lane, human transferrin (10 μg) with anti-human transferrin mAb TH1; right lane, human transferrin (10 μg) with mouse anti-GPD polyclonal antiserum.

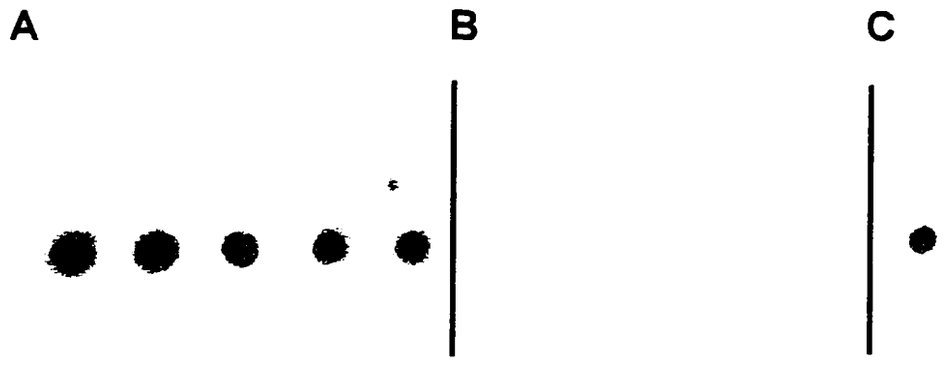

A
B
C

Table 6 Amino acid compositions of *T.b. brucei* 427.01 and *L. donovani* LD3 11 kDa proteins.

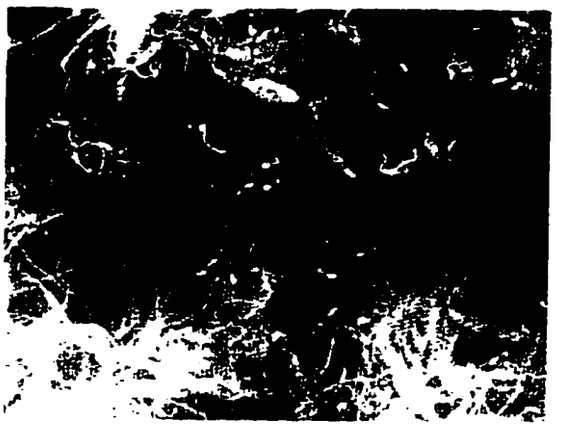
AMINO ACID	COMPOSITION	
	T.b. brucei[a]	*L. donovani*[b]
Asx	12	7
Glx	17	22
Ser	7	5
Gly	12	0
His	2	5
Arg	4	4
Thr	7	4
Ala	11	7
Pro	6	3
Tyr	2	3
Val	7	0
Met	3	5
Ile	3	1
Leu	6	5
Phe	2	8
Lys	7	13

[a]Quadruplicate analyses of the 11 kDa protein eluted from octyl-Sepharose. The composition is based on a molecular mass of 11,453 Da. This value is similar to the apparent molecular mass from SDS-PAGE and to the molecular mass determined for the *Leishmania* KMP-11 molecule [Jardim et al., 1995a; Jardim et al., 1995b].

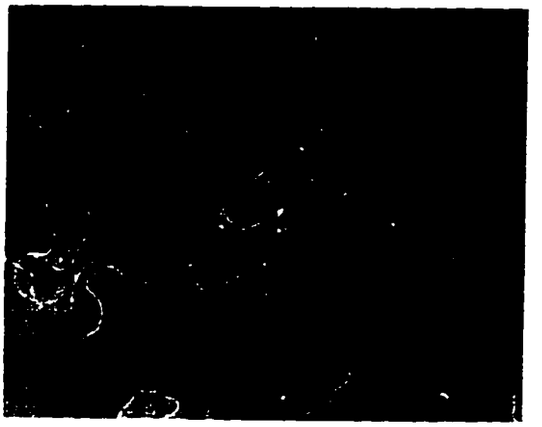
[b]Composition determined by translation from the DNA sequence reported by Jardim et al. [1995b].

Figure 22 Analysis of *Leishmania* and African trypanosomes for 11 kDa protein and procyclin immunoreactivity by immunofluorescence microscopy. **A.** *L. donovani* LD3 promastigotes/mAb L157 (anti-11 kDa protein). **B.** *T. congolense* IL-3000 PCF/mAb L157. **C.** *T.b. rhodesiense* ViTat 1.1 PCF/mAb 247 (anti-*T. brucei* spp. procyclin). **D.** *T. congolense* IL-3000 PCF/mAb 247. E. Confocal laser scanning immunofluorescence profile of *T. congolense* IL-3000 PCF with mAb L157. Magnification 400x.

A

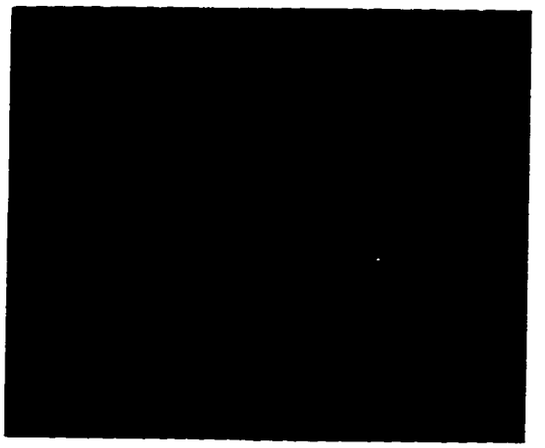


B

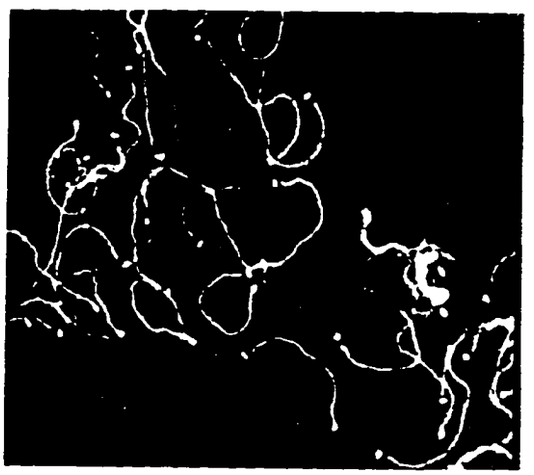


C

D



E



not shown). No binding of the L157 mAb was seen with living or formaldehyde-fixed BSF or PCF (data not shown). The anti-*T. brucei* spp. procyclin mAb 247 was used as a control and showed strong fluorescence on *T.b. rhodesiense* ViTat 1.1 PCF (panel C) and none on *T. congolense* IL3000 PCF (panel D), as expected for a subgenus-specific mAb. Confocal laser scanning immunofluorescence microscopy[12] on acetone-fixed *T. congolense* IL3000 PCF showed a much clearer string-like pattern with bright fluorescent spots at the flagellar base (panel E).

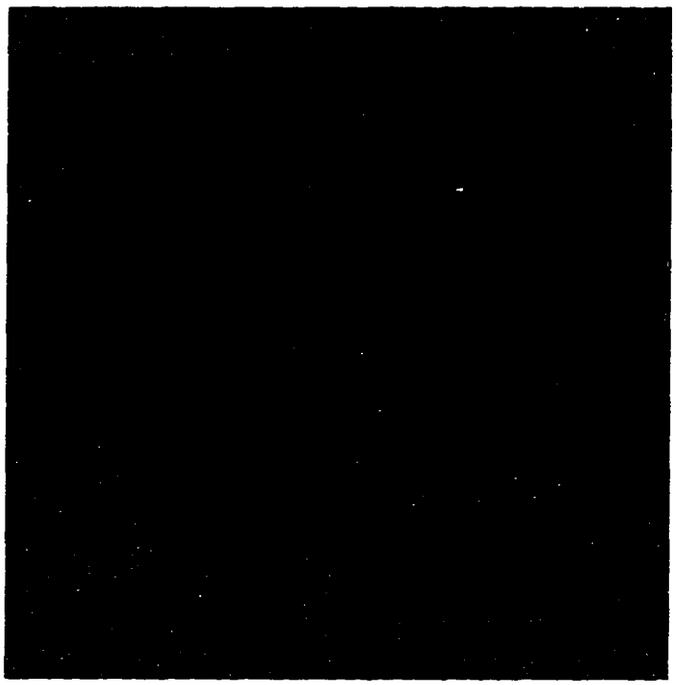
Screening of a T.b. brucei PCF cDNA expression library. The immunological cross-reactivity of the L98/L157 mAb mixture on African trypanosomes suggested its use as a probe in screening a cDNA expression library to obtain the cDNA encoding the trypanosome antigen. One positive plaque was selected (Figure 23, panel A; selected positive plaque indicated by an arrow) and re-screened to verify its positivity (Figure 23, panel B). One positive plaque from the second screen was selected (Figure 23, panel B; selected plaque indicated by an arrow) and the phage DNA was purified (Figure 24, panel A, lane 1). The cDNA insert was amplified by PCR from the purified phage DNA (Figure 24, panel A, lane 2) and was subsequently cloned into the Bluescript™ SK^+ vector (Figure 24, panel A, lane 3) and its nucleotide sequence determined. The 276 bp open reading frame sequence and the corresponding 92 residue translated product are shown in Figure 25. The translated protein has a calculated molecular mass of 10,688 Da, a pI of 6.3 and a net charge of -2 at physiological pH. Comparison of the 92 residue translated product with the *L. donovani* KMP-11 protein sequence identified no sequence similarity between the two proteins, and searching of the protein database with the translated amino acid sequence using the BLAST algorithm revealed no significant sequence similarities to other known proteins.

Overexpression of the 11 kDa trypanosome protein in E. coli. Expression of the 11 kDa trypanosome protein (i.e. the putative KMP-11 molecule identified in the cDNA expression library screen) as a GST-fusion protein was accomplished by cloning the coding region into the pGEX-2T expression vector followed by subsequent expression in *E. coli*. Figure 24, panel B, lane 1 shows the PCR product amplified from *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA using PCR primers designed from the extreme termini of the 11 kDa protein coding region and engineered with restriction sites necessary for cloning into the pGEX-2T vector (Figure 24, panel B, lane 2). Figure 24, panel C,

[12] This work was performed by Roseanne McIndoe, Department of Pathology, University of British Columbia, Vancouver, BC, Canada.

Figure 23 *T.b. brucei* cDNA expression library screen using the L98/L157 mAb mixture. **A.** Primary immunological screen. The immunoreactive plaque selected for secondary screening to verify positivity is indicated by the arrowhead. **B.** Secondary immunological screen. The immunoreactive plaque selected for further analysis is indicated by the arrowhead.

A



B

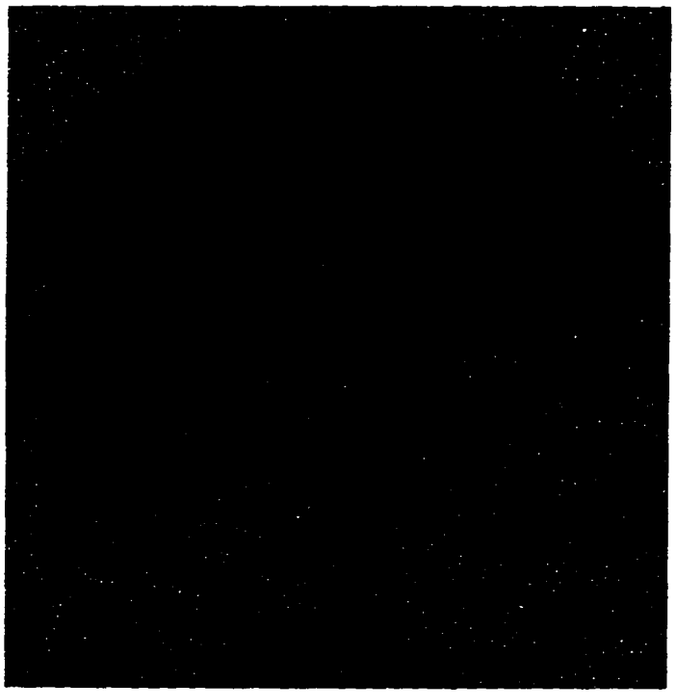


Figure 24 Agarose gel analysis of the immunoreactive phage selected by cDNA expression library screening. **A.** Processing of the immunoreactive phage. Lane 1, isolated phage DNA (upper arrow). Lane 2, PCR amplification of the insert cDNA (lower arrow). Lane 3, amplified PCR fragment cloned into the Bluescript™ SK^+ vector (middle arrow; insert excised with *Not*I and *Sal*I). **B.** Expression vector cloning of the 11 kDa open reading frame. Lane 1, amplified genomic PCR fragment corresponding to the open reading frame (lower arrow). Lane 2, amplified genomic PCR fragment cloned into the pGEX-2T expression vector (upper arrow; insert excised with *Bam*HI and *Eco*RI). For visualization purposes the PCR fragments observed in panels A and B were excised from their respective cloning vectors by digesting with the indicated restriction endonucleases. Molecular size standards (in base pairs) are indicated on the left of each panel. **C.** Heterologous expression of the 11 kDa trypanosome molecule (putative KMP-11) in *E. coli*. Lanes 1 and 2, Coomassie blue stained gel showing lysates of *E. coli* DH5α transformed with the pGEX-2T vector alone or the pGEX-2T-11 kDa trypanosome protein open reading frame construct, respectively. Lanes 3 and 4, immunoblot analysis. Lane assignments are as outlined in lanes 1 and 2, respectively. MAb 6A9 tissue culture supernatant was used as primary antibody at a 1:1 dilution. Protein molecular mass markers (in kilodaltons) are indicated on the left of the panel.

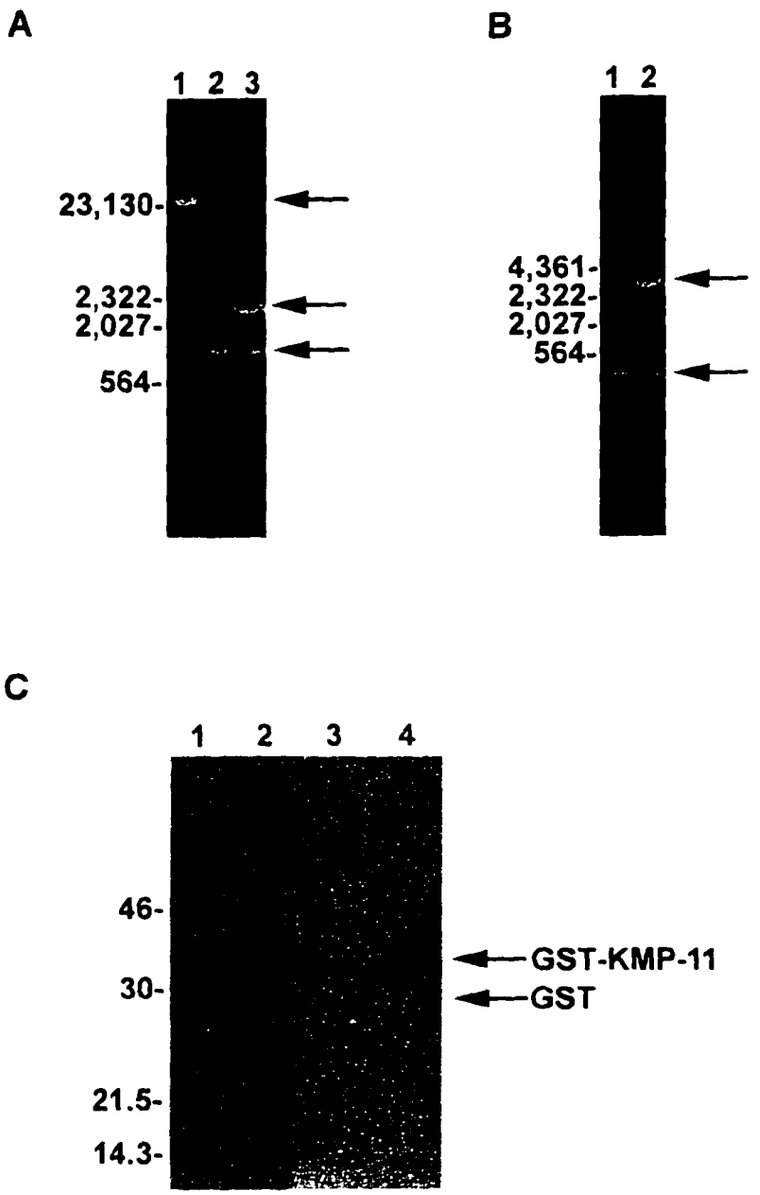

A
1 2 3
23,130-
2,322-
2,027-
564-
B
1 2
4,361-
2,322-
2,027-
564-
C
1 2 3 4
46-
30-
21.5-
14.3-
GST-KMP-11
GST

Figure 25 Nucleotide sequence of the encoding DNA and deduced amino acid sequence of the *T.b. brucei* 11 kDa trypanosome protein identified by the cDNA expression library screen.

* indicates the termination codon.

```
ATGACGAGTA AAAAGATGAG TAATGACTAC AAGAAACACA TGCAAGAGCT ATGGACCCGT    60
M  T  S  K   K  M  S   N  D  Y   K  K  H  M   Q  E  L   W  T  R     20

GTTCTTTTCG TATGTGAATC GACTAACCTC GTCGGTGTAA CGGAAAATGC TACACACCAA   120
V  L  F  V   C  E  S   T  N  L   V  G  V  T   E  N  A   T  H  Q     40

AATTCTCGAC CCGGTACAGA TGAGGAGTTT ATGGCGATTA TTTGGTATCA CTTTCGCCAG   180
N  S  R  P   G  T  D   E  E  F   M  A  I  I   W  Y  H   F  R  Q     60

CACACTTTTT GCCTTTGTCA TCACCCTCCT GGTTTGGTGG TACAGATATG GTCAAGCTCC   240
H  T  F  C   L  C  H   H  P  P   G  L  V  V   Q  I  W   S  S  S     80

TCAATATGCG GAGATGAAGT AAGTTGGTCA CTACTGTAG                          279
S  I  C  G   D  E  V   S  W  S   L  L  *                            92
```

lanes 1 and 2 show Coomassie blue stained lysates of *E. coli* DH5α transformed with the pGEX-2T vector alone or with the pGEX-2T-trypanosome 11 kDa protein construct, respectively, while lanes 3 and 4 show an immunoblot analysis using the L98/L157 mAb mixture on the vector control and potential fusion protein lysates, respectively. The 37 kDa fusion protein (26 kDa GST protein plus 11 kDa trypanosome protein) observed in the DH5α/pGEX-2T-11 kDa protein lysate was absent from the DH5α/pGEX-2T lysate. The fusion protein was detected with the L98/L157 mAb mixture despite the lack of sequence similarity with the *L. donovani* KMP-11 molecule.

Endoproteinase Lys-C digestion of the purified T.b. brucei 11 kDa molecule and amino acid microsequencing.[13] Previous attempts to obtain NH_2-terminal amino acid sequence information from the *T.b. rhodesiense* PCF 11 kDa molecule failed, presumably due to blocked NH_2-termini[14]. To overcome this difficulty an octyl-Sepharose-purified preparation of the 11 kDa molecule was subjected to endoproteinase Lys-C digestion in an attempt to obtain peptides for internal amino acid sequencing. After SDS-PAGE of the Lys-C digest, transfer to Immobilon-P™ and Coomassie blue staining, one peptide band of 5 kDa (data not shown) was selected for gas-phase amino acid microsequencing. A 14 amino acid sequence was obtained (Table 7) which was identical in 12 out of 14 residues spanning amino acids 32-45 of the *L. donovani* KMP-11 sequence. This sequence has been shown to encompass the entire mAb L157 binding site [Jardim et al., 1995a]. It should be noted that this work was performed concurrent with the cDNA expression library screen.

Alternative strategy for isolation of the DNA fragment encoding the trypanosome 11 kDa molecule. Due to the lack of sequence similarity exhibited by the 11 kDa trypanosome molecule identified in the cDNA expression library screen and the conflicting results obtained from amino acid sequencing of the 5 kDa Lys-C-generated peptide, an alternative approach for isolation of the DNA fragment encoding the trypanosome 11 kDa molecule was devised. This strategy selected for the trypanosome 11 kDa molecule that exhibited primary sequence similarity with the *L. donovani* KMP-11 molecule and, as discussed in detail below, involved screening of a *T.b. brucei* cosmid library to obtain 2/3 of the coding sequence, RNA-PCR from *T.b. rhodesiense* poly(A)$^+$ RNA to obtain the

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[13] This work was performed by Gerald Baron, Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada.

[14] R. P. Beecroft and T. W. Pearson, Unpublished results.

remaining 1/3 of the coding sequence and subsequent *T.b. rhodesiense* genomic PCR to obtain the entire coding sequence from one *T. brucei* spp.

The high degree of sequence conservation observed between the internal peptide sequence of the *T.b. brucei* 11 kDa molecule and amino acids 32-45 of the *L. donovani* sequence suggested the use of an *L. donovani* KMP-11 DNA sequence as a heterologous hybridization probe to select for the corresponding *T.b. brucei* gene. A 242 bp DNA fragment of the *L. donovani* KMP-11 coding region (Figure 26, panel A) was used to screen a *T.b. brucei* 427 PCF cosmid library. One positive clone was selected (Figure 26, panel B; selected positive clone indicated by an arrow) and re-screened to verify its positivity (Figure 26, panel C). One positive clone identified in the secondary screen was selected (Figure 26, panel C; selected positive clone indicated by an arrow) and the cosmid DNA was purified from this clone (Figure 27, panel A, lane 1). The large size of the cosmid insert (average of 35-42 kb) necessitated a further step to decrease the insert size for sequencing, and thus the isolated positive cosmid clone DNA was digested with *Eco*RI (Figure 27, panel A, lane 2) and re-probed by Southern blotting with the *L. donovani* KMP-11 probe (lane 3). The probe hybridized to an *Eco*RI fragment of approximately 600 bp, which was subsequently cloned into the pGEM-T™ and Litmus™-39 vectors (Figure 27, panel B, lanes 1 and 2, respectively) for sequencing. This DNA fragment corresponded to approximately 2/3 of the KMP-11 3' coding region as well as a portion of downstream non-coding sequence. To obtain the remaining 5' end of the KMP-11 coding sequence a reverse PCR primer was designed from the 5' region of the 600 bp *Eco*RI cosmid subclone. This primer was used in conjunction with the mini-exon (forward) primer to amplify a 216 bp cDNA fragment corresponding to the NH_2-terminal coding sequence and upstream non-coding sequence by RNA-PCR (Figure 27, panel C, lane 1). This amplified product was cloned into the pGEM-T™ and Litmus™-39 vectors (Figure 27, panel C, lanes 2 and 3, respectively) for sequencing. A 276 bp open reading frame determined from sequencing both the *Eco*RI cosmid subclone and the cDNA PCR fragment was obtained. To confirm the open reading frame sequence from one trypanosome subspecies, a *T.b. rhodesiense* ViTat 1.1 PCF genomic PCR fragment corresponding to the entire coding region of the KMP-11 molecule was amplified (Figure 27, panel D, lane 1). This amplified product was cloned into the pGEM-T™ and Litmus™-39 vectors (Figure 27, panel D, lanes 2 and 3, respectively) for sequencing. Figure 28 shows the nucleotide sequence of the open reading frame and the corresponding translated amino acid sequence. The 92 residue translated product has a calculated molecular mass of 11,078 Da, a pI of 6.1 and a net charge of -2 at

Table 7 NH_2-terminal amino acid sequence of a 5 kDa peptide generated by endoproteinase Lys-C digestion of purified 11 kDa protein from *T.b. brucei*.

CYCLE NUMBER	AMINO ACID RESIDUE
1	A
2	D
3	K
4	P
5	D
6	E
7	A
8	T
9	L
10	S
11	P
12	E
13	M
14	K

Figure 26 *T.b. brucei* cosmid library screen. **A.** The arrowhead indicates the 242 bp DNA fragment encoding amino acids 1-81 of the *L. donovani* KMP-11 molecule that was used as a probe. Molecular size standards (in base pairs) are indicated on the left. **B.** Primary cosmid screen. The hybridizing cosmid clone selected for secondary screening is indicated by the arrowhead. **C.** Secondary cosmid screen. The positive cosmid clone selected for further analysis is indicated by the arrowhead.

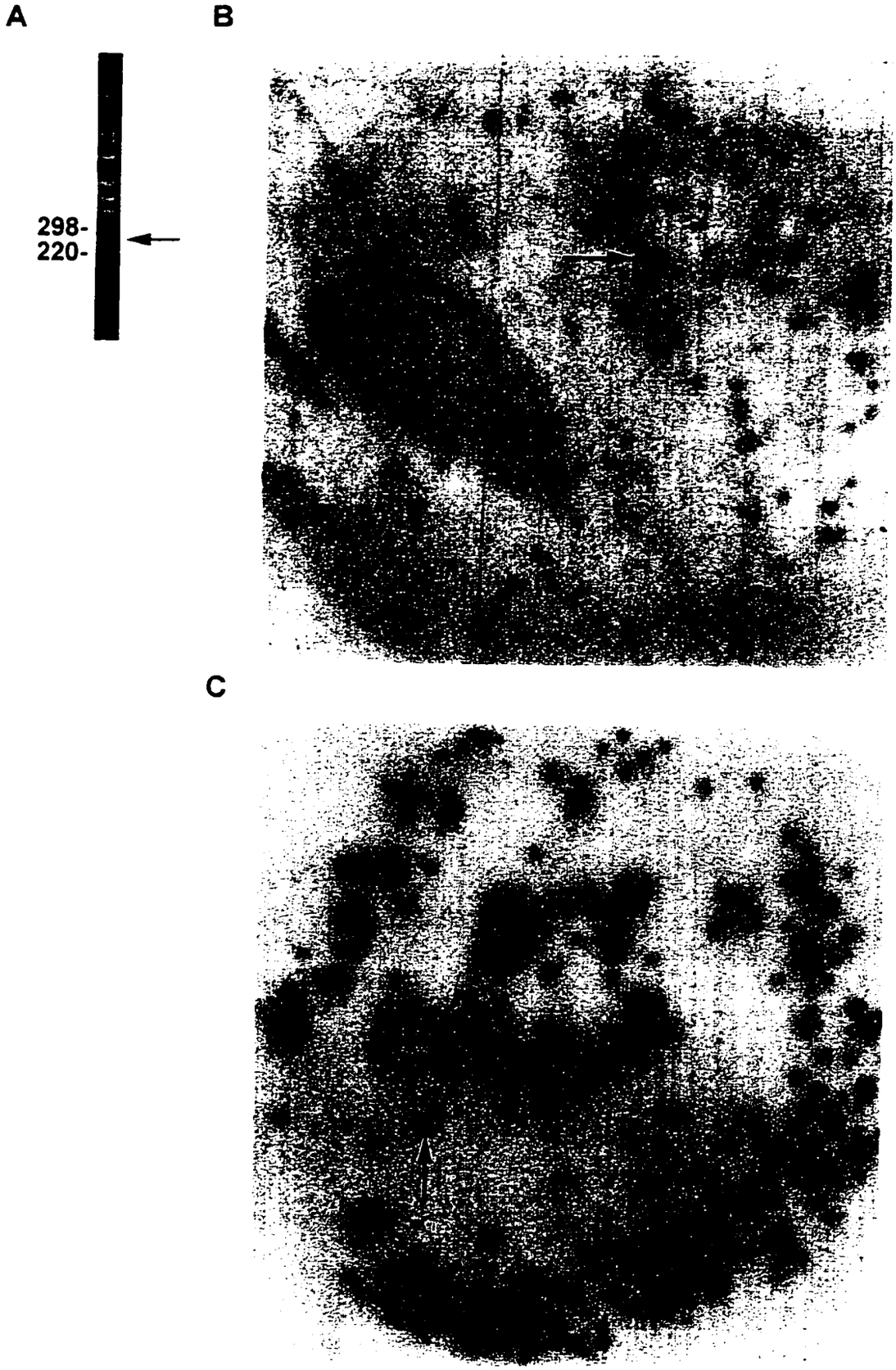

A
B
298-
220-
C

Figure 27 Agarose gel analyses showing isolation of the gene encoding the trypanosome 11 kDa molecule identified by the cosmid library screen. For visualization purposes PCR fragments were excised from their respective cloning vectors by digesting with the indicated restriction endonucleases. Molecular size standards (in base pairs) are indicated on the left of each panel. **A.** Processing of the *T.b. brucei* positive cosmid clone. Lane 1, purified cosmid DNA. Lane 2, *Eco*RI digest probed with the 242 bp *L. donovani* KMP-11 gene fragment. A hybridizing DNA fragment of approximately 600 bp was selected for further analysis (indicated by the arrow). **B.** The 600 bp *Eco*RI hybridizing DNA fragment (lower arrow) subcloned into either the pGEM-T™ vector (lane 1; upper arrow; excised with *Nco*I and *Nde*I) or the Litmus-39™ vector (lane 2; upper arrow; excised with *Sal*I and *Sph*I). **C.** Lane 1, the *T.b. rhodesiense* 216 bp PCR fragment (lower arrow). Lanes 2 and 3, the 216 bp PCR fragment cloned into either the pGEM-T™ vector (upper arrow; excised with *Nco*I and *Nde*I) or the Litmus-39™ vector (upper arrow; excised with *Sal*I and *Sph*I), respectively. **D.** Lane 1, the *T.b. rhodesiense* genomic PCR fragment corresponding to the KMP-11 coding region (lower arrow). Lanes 2 and 3, the genomic PCR fragment cloned into either the pGEM-T™ vector (upper arrow; excised with *Nco*I and *Nde*I) or the Litmus-39™ vector (upper arrow; excised with *Sal*I and *Sph*I), respectively.

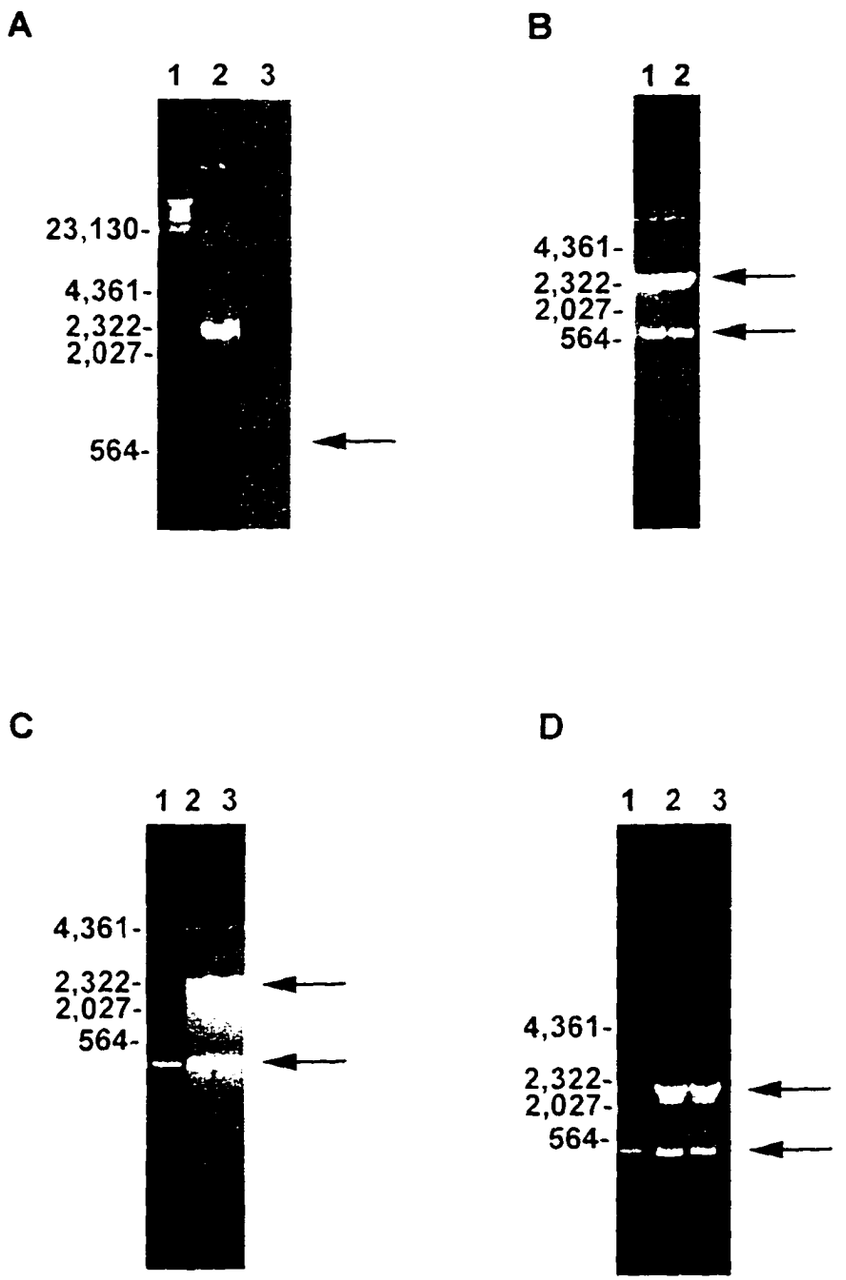

A
1 2 3
23,130-
4,361-
2,322-
2,027-
564-
B
1 2
4,361-
2,322-
2,027-
564-
C
1 2 3
4,361-
2,322-
2,027-
564-
D
1 2 3
4,361-
2,322-
2,027-
564-

Figure 28 Nucleotide sequence of the encoding DNA and deduced amino acid sequence of the *T.b. rhodesiense* 11 kDa protein identified by the cosmid library screen. Amino acids 32-45 corresponding to the internal sequence obtained from gas-phase microsequencing of the 5 kDa Lys-C-generated 11 kDa peptide are underlined by a single line. These residues also correspond to the region containing the mAb L157 epitope. The sequence corresponding to a region containing the mAb L98 epitope (amino acids 72-92) is underlined by a double line [Jardim et al., 1995a].

* indicates the termination codon.

```
ATGGCCACCA CATACGAAGA ATTTGCTGCG AAGCTCGACC GCCTCGATGC CGAATTCGCC    60
M  A  T  T   Y  E  E   F  A  A   K  L  D  R   L  D  A   E  F  A     20

AAGAAGATGG AGGAGCAGAA CAAGCGATTC TTCGCTGACA AGCCTGATGA GGCTACGCTG   120
K  K  M  E   E  Q  N   K  R  F   F  A  D  K   P  D  E   A  T  L     40

TCCCCTGAGA TGAAAGAGCA CTATGAAAAG TTCGAAAAAA TGATCCAGGA GCACACGGAC   180
S  P  E  M   K  E  H   Y  E  K   F  E  K  M   I  Q  E   H  T  D     60

AAGTTCAACA AGAAGATGCG CGAGCACTCA GAGCACTTCA AGGCCAAGTT TGCGGAACTC   240
K  F  N  K   K  M  R   E  H  S   E  H  F  K   A  K  F   A  E  L     80

CTCGAGCAGC AGAAGAATGC CCAGTTCCCC GGAAAATGA                          279
L  E  Q  Q   K  N  A   Q  F  P   G  K  *                            92
```

physiological pH. The internal amino acid sequence obtained by protein microsequencing of the 5 kDa Lys-C-generated 11 kDa peptide matched the translated sequence at positions 32-45 (Figure 28).

A search of the protein database with the translated amino acid sequence using the BLAST algorithm identified KMP-11 from *L. donovani* [Jardim et al., 1995b] as the optimal scoring protein and revealed no significant sequence similarities to other known proteins. The alignment of the KMP-11 amino acid sequences from *T. brucei* and *L. donovani* is shown in Figure 29. The KMP-11 molecules of the related kinetoplastids showed 82% sequence similarity. It is of interest that the lysine at position 45 of the trypanosome sequence was a modified amino acid (monomethylarginine) in the leishmania molecule (see discussion).

KMP-11 secondary structure. The KMP-11 secondary structure was predicted by analysis of the protein's primary sequence using the algorithm of Garnier et al. [1978] which predicts protein secondary structure solely from the amino acid sequence. This algorithm assigned a high α-helical content of 88% and predicted that the trypanosome KMP-11 would assume a helix-turn-helix motif. Two helices of 31 and 50 residues each were predicted to be separated by a random-coil segment followed by a second random-coil at the C-terminus. An α-helical wheel plot of this motif shows it to be amphipathic, with approximately one-third of one face of each helix composed predominantly of hydrophobic residues and the remainder of the helices consisting mainly of charged residues. A schematic representation of this predicted structure is shown in Figure 30.

KMP-11 gene copy number and mRNA transcript size. The KMP-11 gene copy number was determined by Southern blot analysis of restriction endonuclease digests of *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA. The blots were probed with the homologous KMP-11 gene. A variety of restriction endonucleases were selected for this analysis, including two which contained cleavage sites within the KMP-11 gene (*Eco*RI, *Xho*I), and several for which cleavage sites were not found within the gene (*Bam*HI, *Hind*III, *Kpn*I, *Nde*I, *Sau*3AI). Figure 31, panel A shows a diagram of the restriction endonuclease map for the KMP-11 gene. As shown in Figure 31, panel B the *Eco*RI and *Xho*I digests each showed two DNA fragments that hybridized to the KMP-11 probe, while the remaining digests produced only a single hybridizing DNA fragment. These results suggest the presence of one gene copy encoding the trypanosome KMP-11. The homologous KMP-11 probe was also used in Northern blot analysis and was found to

Figure 29 The deduced amino acid sequence of the *T.b. rhodesiense* KMP-11 and comparison with the corresponding sequence from *L. donovani* [Jardim et al., 1995b]. Sequence identities are indicated by boxed regions.

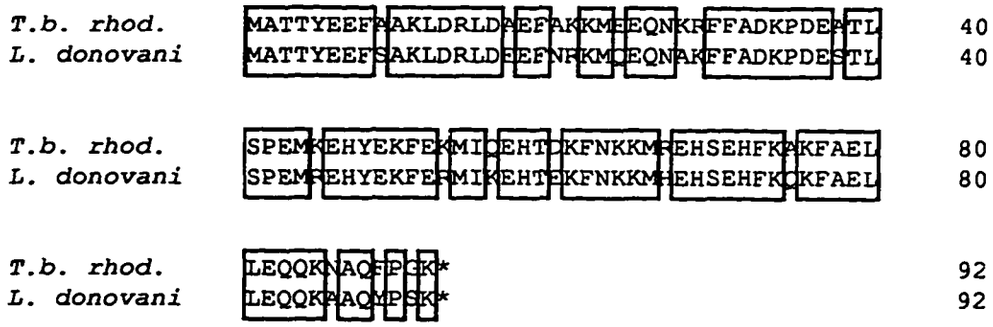

T.b. rhod. MATTYEEFSAKLDRLDEEFAKKMEEQNKRFFADKPDESTL 40
L. donovani MATTYEEFSAKLDRLDEEFNRKMQEQNAKFFADKPDESTL 40
T.b. rhod. SPEMKEHYEKFEKMIQEHTQKFNKKMREHSEHFKAKFAEL 80
L. donovani SPEMREHYEKFERMIKEHTEKFNKKMHEHSEHFKQKFAEL 80
T.b. rhod. LEQQKNAQFPGK* 92
L. donovani LEQQKAAQMPSK* 92

Figure 30 A schematic representation of the predicted secondary structure of *T.b. rhodesiense* KMP-11. The circled residues indicate hydrophobic amino acids. The shaded regions indicate the approximate hydrophobic face of the KMP-11 helices (diagram template taken from Jardim et al., 1995b].

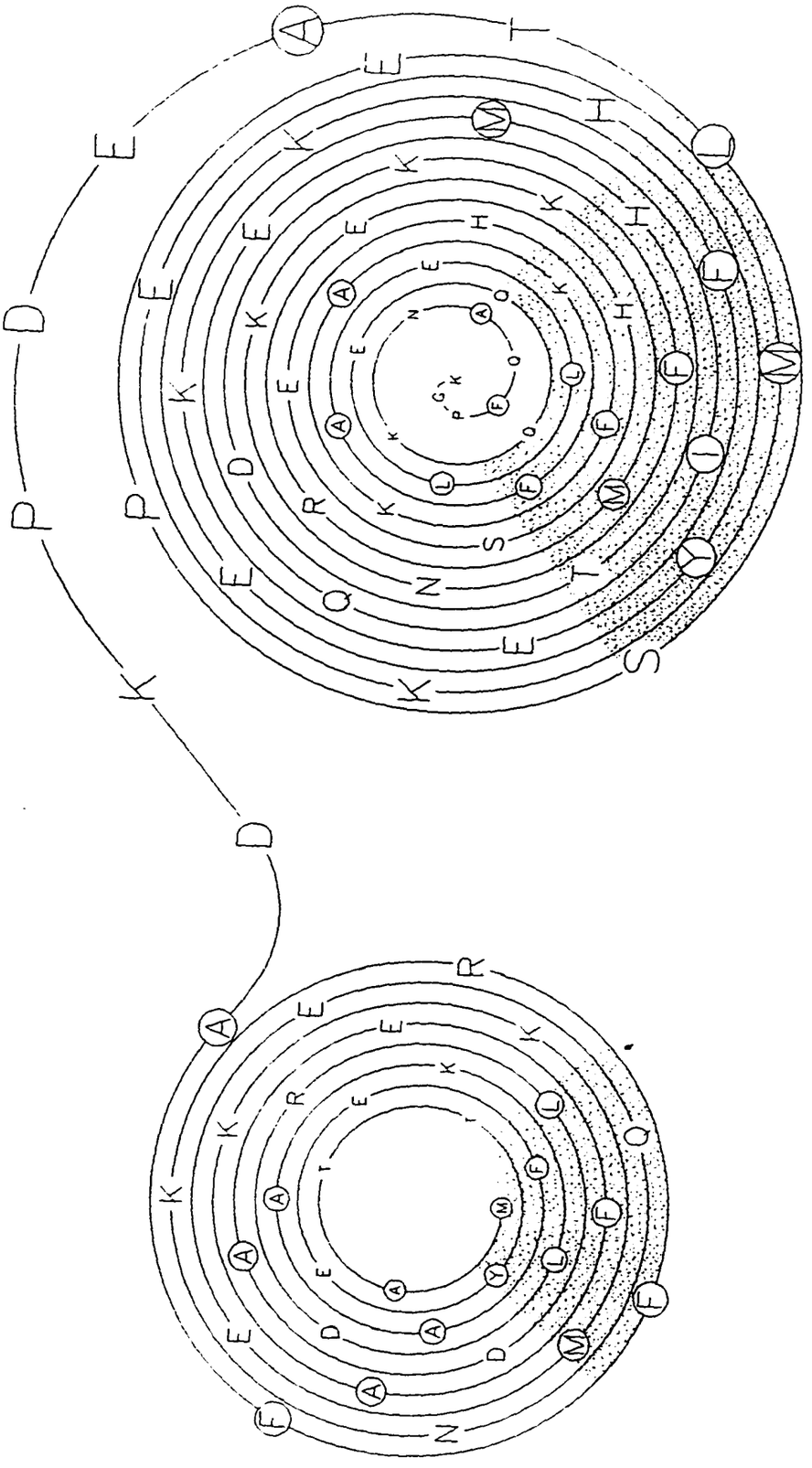
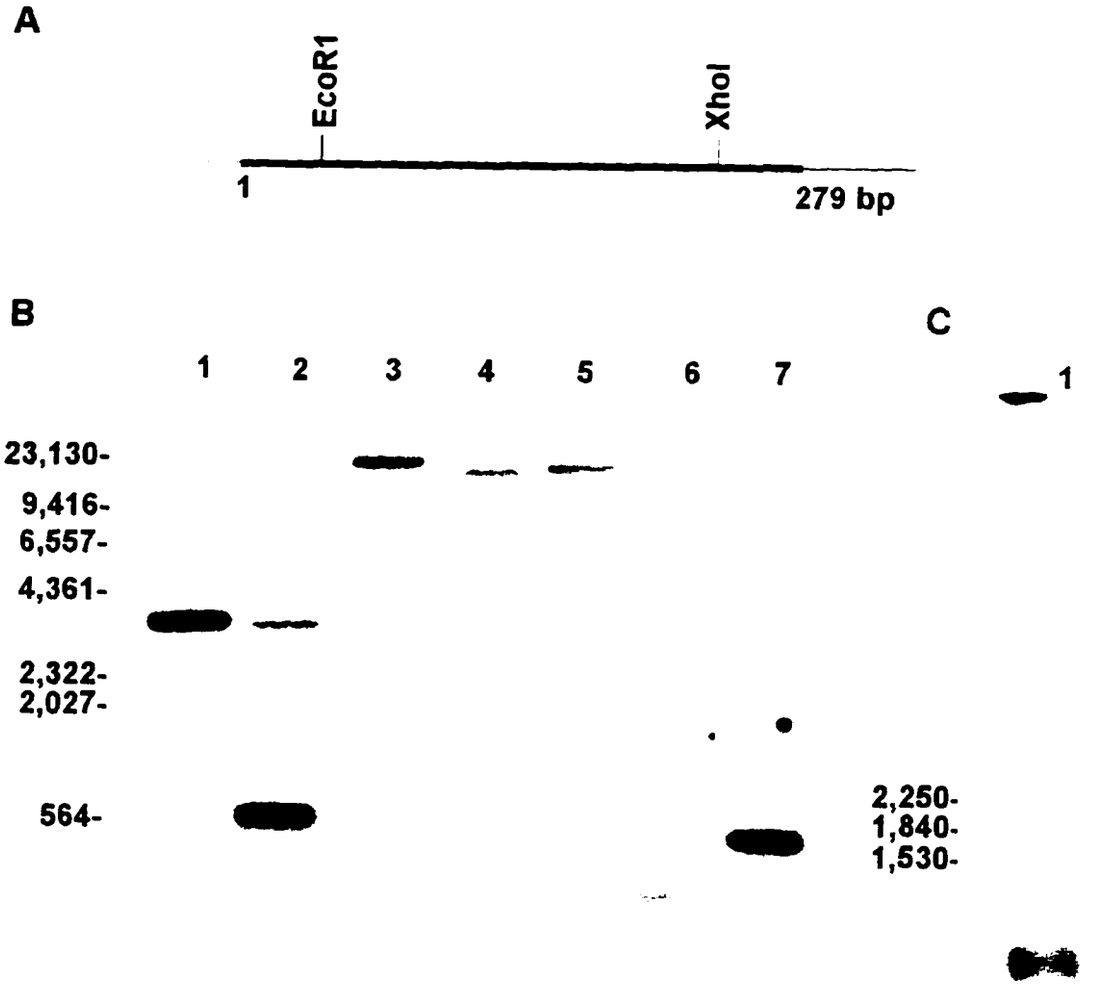


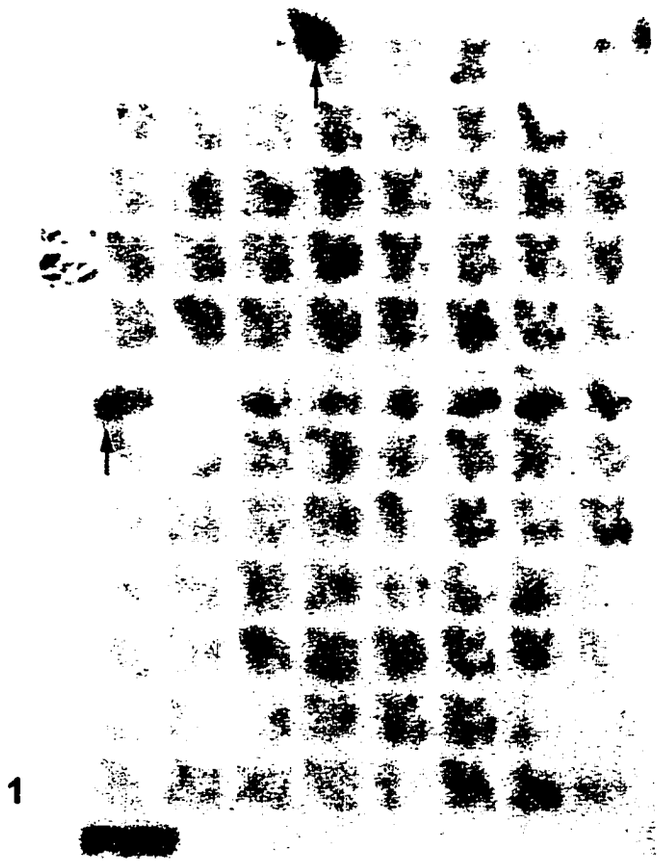
Figure 31 Analysis of the KMP-11 gene copy number and expression of the mRNA transcript by Southern and Northern blots. **A.** Restriction endonuclease map for the 279 bp *T.b. rhodesiense* KMP-11 coding region. Only restriction sites used in the Southern blot analysis are indicated. **B.** Southern blot analysis of *T.b. rhodesiense* ViTat 1.1 PCF genomic DNA probed with the homologous KMP-11 gene. Lanes 1 to 7 represent genomic DNA digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Nde*I, *Sau*3AI and *Xho*I, respectively. **C.** Northern blot analysis of *T.b. rhodesiense* ViTat 1.1 PCF poly(A)$^+$ RNA probed with the homologous KMP-11 gene. Molecular size standards (in base pairs) are indicated on the left of panels B and C.


A
EcoR1
XhoI
1
279 bp
B
1
2
3
4
5
6
7
23,130-
9,416-
6,557-
4,361-
2,322-
2,027-
564-
C
1
2,250-
1,840-
1,530-

hybridize to a single KMP-11 mRNA transcript of approximately 800 bases (Figure 31, panel C, lane 1).

Bacteriophage P1 library screen. To obtain genomic DNA sequence flanking the KMP-11 gene a bacteriophage P1 library high density filter representative of the leishmania and trypanosome genomes was screened with the 279 bp trypanosome genomic KMP-11 probe. The filter colony layout is shown in Figure 15. The screen identified one *L. donovani* colony and one *T. brucei* colony that hybridized with the probe, and these colonies were selected for further analysis (Figure 32; selected positives indicated by arrows). DNA was isolated from each of these positive clones and rescreened by dot blotting to verify positivity. The *T. brucei* positive clone was subjected to restriction endonuclease digestion, reprobed by Southern blotting with the trypanosome KMP-11 probe and one well-isolated hybridizing DNA fragment of approximately 10,000 bp was selected (data not shown). This fragment size is substantially larger than the KMP-11 open reading frame (276 base pairs), and thus will probably contain large stretches of genomic flanking sequences that can be used to design plasmid constructs for knockout mutagenesis (this work is currently in progress) and is beyond the scope of this thesis.

Figure 32 Autoradiograph of the bacteriophage P1 trypanosome/leishmania library high density filter probed with the 279 bp KMP-11 gene from *T.b. rhodesiense* ViTat 1.1 PCF. The hybridizing clones that were selected for further study are indicated by arrows (arrow at the top of the filter indicates the hybridizing trypanosome clone, while the arrow on the middle left corresponds to the hybridizing leishmania clone). To facilitate identification of hybridizing clones the filter is oriented such that the letters A to H extend from left to right and the numbers 1 to 12 extend from bottom to top (for simplicity only the letter A and the number 1 are indicated on the diagram).


1
A

DISCUSSION

This chapter reports the molecular characterization of an 11 kDa plasma membrane protein purified from *T.b. rhodesiense* and analysis of its encoding gene. This trypanosomal protein was originally detected [Tolson et al., 1989] by its immunological reactivity with mAbs specific for what has historically been called the leishmania lipophosphoglycan-associated protein [Jardim et al., 1991]. The results reported here show that an immunologically cross-reactive 11 kDa protein is present throughout a variety of kinetoplastid parasites, including African trypanosomes, *Leishmania*, *Trypanosoma cruzi*, *Crithidia fasciculata*, *Leptomonas collosma* and *Phytomonas* spp. It is interesting that the abundance of these molecules appeared to be much higher in the trypanosomes and *Leishmania*, since 10-fold more parasites was required to obtain equivalent signals in immunoblots with the other species. However, it is possible that the immunoreactivity of the molecule is simply weaker in these species or that the molecules are less accessible. The 11 kDa protein was not found in mammalian cells nor in several non-kinetoplastids examined. It was therefore proposed in published work resulting partly from this thesis that the 11 kDa proteins comprise a set of molecules that may differ slightly in different kinetoplastid parasites and suggest that this set of molecules be given the name kinetoplastid membrane protein-11 (KMP-11) [Tolson et al., 1994a; Stebeck et al., 1995]. This would allow the specific designation of the molecules as *T.b. rhodesiense* KMP-11, *L. donovani* KMP-11, *T. cruzi* KMP-11 etc.

The original observation that African trypanosomes contained epitopes immunologically cross-reactive with the leishmania protein was made in immunofluorescence studies using acetone-permeabilized *T. congolense* [Tolson et al., 1989]. In this chapter these studies have been extended by immunoblot analysis which showed that all six species or subspecies of African trypanosomes examined express these epitopes on an 11 kDa protein. The level of expression was fairly uniform throughout these parasites with the exception of *T.b. brucei* 427.01 PCF which appeared to contain about half as much as the others, indicating that clone-specific levels of expression occur. In addition, the trypanosome KMP-11 molecule was found to be expressed throughout the life cycle of *T. congolense* IL3000, although at much lower levels in BSF than in PCF, epimastigotes or even metacyclic forms. The protein was clearly present in DEAE-purified BSF parasites and is thus not confined to insect stages. Upon differentiation from BSF to PCF, expression was increased within 2 hours of triggering differentiation. Expression of the trypanosome KMP-11 molecule occurred

earlier and plateaued earlier than that of the *T. congolense* IL-3000 PCF procyclin, the glutamic acid/alanine-rich protein (GARP). It is clear, however, that the increased expression of the trypanosome KMP-11 molecule coincided approximately with GARP expression and occurred within hours of initiating transformation, prior to any observable morphologic changes [Stebeck et al., 1995]. It is also apparent that the KMP-11 molecule is stage-regulated in *T. cruzi*, since epimastigotes expressed much higher levels than trypomastigotes. In addition, such stage-regulated expression has been observed for *L. donovani* KMP-11, as the level of expression is reduced in amastigotes when compared to promastigotes[15]. Why insect stages of the various kinetoplastid parasites express more of the KMP-11 molecule than life cycle stages found in the mammalian host is not understood, but may relate to the function of this molecule in the various parasites (discussed below).

The strategy used to purify the leishmania KMP-11 molecule from *L. donovani* promastigotes [Jardim et al., 1991] was extended to procyclic trypanosomes to purify the corresponding trypanosome KMP-11 molecule. Organic solvent extraction and reverse-phase HPLC using an octyl-Sepharose column resulted in elution of the purified trypanosome KMP-11 molecule at the same position as the leishmania KMP-11 molecule. The procyclin eluted later in the elution gradient, a situation somewhat analogous to that observed with *Leishmania* where both the KMP-11 molecule and LPG elute sequentially from octyl-Sepharose [Jardim et al., 1991]. It remains to be determined if the co-isolation of the KMP-11 molecules and the other major surface molecules (PARP, GARP, LPG) is a result of the molecules associating *in situ* in the cell membrane or as a result of the isolation procedure or simply reflects their solubility characteristics, since the KMP-11 molecules are amphipathic (discussed in more detail below).

The KMP-11 molecule was found to be present in plasma membrane-enriched preparations from *T.b. rhodesiense* and thus the accessibility of the KMP-11 molecule on the trypanosome surface was tested by the use of cell-surface labeling techniques. Surface labeling with two different biotinylation reagents (NHS-SS-biotin and sulfo-NHS-biotin) failed to detect an 11 kDa molecule on the surface of living cells. Immunofluorescence using the L98/L157 mAb mixture on living and on formaldehyde-fixed trypanosome PCF was also consistently negative. These results parallel those observed in *Leishmania* where cell surface biotinylation failed to label an 11 kDa

[15] A. Jardim and R. W. Olafson, personal communication.

molecule[15] and no immunofluorescence was detected on living *Leishmania* promastigotes with the L98/L157 mAb mixture [Stebeck et al., 1995]. Cell surface iodination experiments performed on *L. donovani* promastigotes have, however, demonstrated labeling of an 11 kDa molecule [Jardim et al., 1995a]. In dot blotting experiments reported in this thesis L98/L157 immunoreactivity was found almost exclusively in the detergent phase of *T.b. rhodesiense* Triton X-114 extracts. Taken together, these results suggest that the KMP-11 molecule is membrane-associated but inaccessible to cell surface biotinylation reagents or antibodies, either as a consequence of non-cell surface disposition (in trypanosomes) or as a result of shielding imparted by tight association with the procyclins, PARP and GARP, on procyclic trypanosomes or LPG on leishmania. Future studies involving co-capping, as was performed to verify the *in vivo* association of the gene B protein with LPG on *L. major* promastigotes (see introduction), may help to resolve the surface disposition of these molecules.

The fluorescence pattern determined by indirect and confocal laser scanning immunofluorescence microscopy on trypanosomes was shown to extend along the length of the flagellum with a concentrated region of fluorescence at the flagellar base in the region called the 'reservoir' by Vickerman [1970]. This localized immunoreactivity suggests the trypanosome KMP-11 molecule may be associated with the flagellum and the flagellar pocket. A similar localization of immunofluorescence was observed for leishmania, but in addition these kinetoplastids also exhibited a more widespread pattern, thus suggesting the leishmania KMP-11 molecule may perform a slightly different role in these parasites.

Further characterization of the trypanosome KMP-11 molecule revealed that it was non-glycosylated and present on the procyclic trypanosome membrane at 2×10^5 - 1×10^6 molecules per cell. This high copy number makes the KMP-11 molecule the third most abundant molecule characterized on the trypanosome membrane to date, with the other two being the stage-specific major cell surface coat proteins VSG and the procyclins, PARP and GARP. Previous investigations of the leishmania KMP-11 molecule showed that it was glycosylated and present on promastigotes at 1×10^6 - 2×10^6 molecules per cell [Jardim et al., 1995a]. The differences between the KMP-11 molecules from *L. donovani* and *T.b. rhodesiense* were further exemplified by amino-acid microanalysis of the two molecules which showed that the proportions of amino acids differed between them.

Determination of the magnitude of sequence divergence between the KMP-11 molecules of the related kinetoplastids necessitated isolation of the gene encoding the trypanosome KMP-11 molecule. The initial gene isolation attempt used the L98/L157 mAb mixture to screen a *T.b. brucei* cDNA expression library and produced interesting results. The cDNA selected from a serologically positive phage clone contained a 276 base pair open reading frame encoding a 92 amino acid translated protein product. This is the same number of amino acids found in the leishmania KMP-11 molecule reported by Jardim et al. [1995b]. Expression of this trypanosome DNA as a GST-fusion protein in *E. coli* generated a recombinant protein that was immunologically cross-reactive with the mAb L98/L157 mixture. However, despite this immunological cross-reactivity, there was no observable sequence conservation within the mAb L98/L157 binding sites determined for the *L. donovani* molecule [Jardim et al., 1995b]. In addition, there was no significant overall sequence similarity detected between the *T.b. brucei* translated protein and the leishmania KMP-11 molecule. This seemingly paradoxical result may be explained by the formation of a cross-reactive conformational epitope within the expressed protein that would not be apparent from the primary sequence. The observed immunoreactivity may have been purely artifactual. However, the fact that the translated product is of exactly the same number of amino acids as the leishmania KMP-11 molecule suggests the possibility that this trypanosome protein is functionally related to the KMP-11 molecules and is worth examining in this regard.

The octyl-Sepharose-purified trypanosome KMP-11 molecule was subjected to endoproteinase Lys-C digestion to obtain internal amino acid sequence information from the 11 kDa molecule. The 14 residue sequence obtained encompassed the mAb L157 binding site and was found to differ from the corresponding leishmania KMP-11 sequence in only 2 out of the 14 residues. This high degree of sequence conservation suggested the use of the leishmania KMP-11 gene as a heterologous probe to screen a trypanosome cosmid library as an alternative approach for isolation of the gene encoding the corresponding trypanosome molecule. This screen once again selected a positive clone containing an open reading frame of 276 base pairs and encoding a 92 amino acid translated protein; however, in this instance the leishmania and trypanosome KMP-11 molecules exhibited 82% sequence conservation. The most highly conserved sequences localized to the mAb L98 and L157 epitopes, which encompass amino acid residues 72-92 and 32-45 respectively in the *L. donovani* molecule [Jardim et al., 1995a].

Assuming that the positive plaque identified by screening the cDNA expression library with mAbs was a true positive, the isolation of two cDNA fragments encoding translated products of 92 residues is suggestive of the existence of a family of 11 kDa proteins in trypanosomes. In support of this theory, a total of four plaques were found to exhibit immunoreactivity with the L98/L157 mAb mixture. None of the three additional positive plaques were characterized further.

Comparison of the *L. donovani* and *T.b. rhodesiense* KMP-11 sequences highlighted a key divergent amino acid residue, the lysine residue found at position 45 of the trypanosome sequence. An N^G-monomethylarginine residue was found at this position of the *Leishmania* sequence, and has been suggested to play a role in the survival of *Leishmania* parasites within the phagolysosomes of infected macrophages [Jardim et al., 1995a]. Nitric oxide has been implicated as the central leishmanicidal agent in activated murine macrophages, and monomethylarginine treatment of macrophages was shown to inhibit nitric oxide synthase allowing intracellular parasite proliferation [Liew et al., 1990; Lawrence and Robert-Gero, 1993]. It was proposed by Jardim et al. [1995a] that within the acidic environment of the phagolysosome KMP-11 is released from the parasite and degraded to its constituent amino acids, of which monomethylarginine would subsequently act to ensure parasite survival. The extracellular growth of African trypanosomes would alleviate the requirement for such a survival mechanism, thus possibly explaining the replacement of this amino acid with lysine in the trypanosome KMP-11 sequence.

The secondary structure of the trypanosome KMP-11 molecule was predicted by applying the Garnier algorithm [Garnier et al., 1978] to the primary sequence. This algorithm predicted that the KMP-11 molecule forms a helix-turn-helix motif, and an α-helical wheel plot of this motif generated two amphipathic helices connected by a random-coil segment. Approximately one-third of one face of each helix consists almost entirely of hydrophobic residues, suggesting a potential interaction with lipid bilayers, while the remainder of the helices consist largely of charged, hydrophilic residues. An analogous representation and interpretation has been made for the *L. donovani* KMP-11, and evidence supporting the KMP-11-lipid bilayer interaction has been provided by *L. donovani* KMP-11-mediated carboxyfluorescein release from liposomes [Jardim et al., 1995b].

As previously discussed, the tight association and co-isolation reported for the *L. donovani* KMP-11 molecule with LPG of promastigotes [Jardim et al., 1991] mirrors the situation observed with procyclic African trypanosomes where KMP-11 has invariably been found to co-isolate with the major cell surface glycoproteins, the procyclins. The results presented in this chapter suggest that the KMP-11 molecules from leishmania and trypanosomes share a similar membrane association but may differ in their disposition and distribution. The *L. donovani* KMP-11 has been purported to be involved in stabilizing LPG within the parasite membrane by regulating the overall lipid bilayer pressure via a putative membrane association with LPG [Jardim et al., 1995b]. The cell copy number of the leishmania KMP-11 molecule ($1 - 2 \times 10^6$ molecules per cell) lends support to this theory, as it is similar to the copy number of LPG on the *L. donovani* promastigote membrane [Jardim et al., 1995a]. The trypanosome KMP-11, however, appears to be much more localized in the cell and no evidence exists that it is surface exposed. The trypanosome molecule may thus not function to stabilize molecules within the membrane. The cell copy number observed for the trypanosome KMP-11 molecule ($2 \times 10^5 - 1 \times 10^6$ molecules per cell) supports this rationale, as it is significantly lower than that observed for procyclin on the procyclic trypanosome membrane (6×10^6 molecules per cell) [Clayton and Mowatt, 1989]. It is apparent from results presented in this chapter that KMP-11 molecules may be of particular functional importance to kinetoplastid life cycle stages found within the insect vectors, as their expression is upregulated in *L. donovani* promastigotes, *T.b. rhodesiense* PCF and *T. cruzi* epimastigotes, and this upregulation occurs in concert with upregulation of the major cell surface molecules found on such trypanosome and leishmania life cycle stages. Consequently, it can be hypothesized that at some level this molecule may function, either directly or indirectly, in vector-parasite interactions. However, the functional role of the KMP-11 molecule in various kinetoplastid parasites must await further studies.

Southern blot analysis revealed one gene copy for KMP-11 in the *T.b. rhodesiense* ViTat 1.1 clone, in stark contrast to the three gene copies reported for *L. donovani* KMP-11 [Jardim et al., 1995b]. This low gene copy number suggests that the trypanosome KMP-11 may be an ideal candidate for attempts at gene knockout mutagenesis. The abundance of KMP-11 in procyclic trypanosomes is approximately two fold less than that found for *Leishmania* promastigotes (see discussion above). This decreased abundance may be explained by the single KMP-11 gene copy found in the *T.b. rhodesiense* ViTat 1.1 trypanosomes, as compared to the three gene copies in the *L. donovani* IS2D clone [Jardim et al., 1995b]. Nevertheless, the single gene copy and high

level of protein expression suggest that the trypanosome KMP-11 gene may be under the control of a highly active promoter. Northern blot analysis detected a single KMP-11 transcript of approximately the appropriate size (~800 bases) to encode the 92 amino acid protein, suggesting that no major post-transcriptional modifications occur to alter the size of the KMP-11 transcript prior to translation. Studies investigating the level of expression of the KMP-11 molecule throughout the trypanosome life cycle revealed approximately equal levels in *T. congolense* IL3000 PCF, epimastigotes and metacyclics and significantly reduced expression in bloodstream forms (BSF). These results indicate that KMP-11 expression is differentially regulated between bloodstream- and insect-stages of trypanosomes, although it is currently unknown if the regulation is of a transcriptional or a translational nature.

Previous investigations have demonstrated that in both *Leishmania* and *Trypanosoma* the KMP-11 molecule is a potent stimulator of T cells [Tolson et al., 1994a]. In these studies promastigotes from a wide variety of *Leishmania* species and procyclic African trypanosomes were shown to stimulate proliferation of *L. donovani* KMP-11-primed or *L. donovani* promastigote-primed lymph node cells. Although these findings are seemingly more relevant to Leishmania due to the intracellular life style of these parasites, the functional significance to bloodstream form trypanosomes should not be overlooked as immunity against this protozoan is poorly understood. Protective immunity against trypanosomiasis has historically been believed to be mediated by the humoral arm of the immune response, with minor involvement of cell-mediated immunity. These assumptions have been derived from investigating active trypanosome infections which are typically characterized by suppression of T cell-mediated responses [Vickerman et al., 1993]. The immunological benefit of mounting an effective helper T cell response to a conserved molecule on the trypanosome membrane would be at least threefold. First, the elaboration of cytokines resulting from induction of such cell-mediated immunity may prove favorable and aid in thwarting disease progression. Second, the production of a cell-mediated immune response may serve to activate B cells and thus generate a faster and more effective generalized humoral response early in infection to eliminate parasitemia. Third, the induction of T cell help may specifically induce a memory-generating anti-KMP-11 IgG response. In order for the latter immune response to succeed in reducing parasitemia the trypanosome KMP-11 molecule would have to be present on the external face of the plasma membrane, thus further highlighting the need for definitive localization of this molecule's membrane disposition. Additionally, the KMP-11 molecules would have to be made accessible through the VSG surface coat,

a scenario which could result from antibody cross-linking of the VSG molecules on the cell surface.

What is the biological function of the KMP-11 molecules? A direct attempt to determine the function within the trypanosome is currently underway by performing knockout mutagenesis of the KMP-11 molecule. This will entail attainment of genomic sequences flanking the KMP-11 gene by screening of a bacteriophage P1 library representative of the trypanosome genome, with subsequent use of these flanking sequences in the construction of plasmid constructs to facilitate mutagenesis in a tsetse fly-transmissible trypanosome strain that sports a low KMP-11 gene copy number. To obtain a more integrated picture of KMP-11 function, future work will also utilize a similar strategy to perform knockout mutagenesis of KMP-11 in a sandfly-transmissible *Leishmania* strain.

MUCH OF THE WORK OUTLINED IN THIS CHAPTER HAS BEEN PUBLISHED:

Tolson, D. L., Jardim, A., Schnur, L. F., Stebeck, C. E., Tuckey, C., Beecroft, R. P., Teh, H., Olafson, R. W. and Pearson, T. W. 1994. The kinetoplastid membrane protein 11 of *Leishmania donovani* and African trypanosomes is a potent stimulator of T-lymphocyte proliferation. Infection and Immunity. 62: 4893-4899.

Stebeck, C. E., Beecroft, R. P., Singh, B. N., Jardim, A., Olafson, R. W., Tuckey, C., Prenevost, K. D. and Pearson, T. W. 1995. Kinetoplastid membrane protein-11 (KMP-11) is differentially expressed during the life cycle of African trypanosomes and is found in a wide variety of kinetoplastid protozoan parasites. Molecular and Biochemical Parasitology. 71: 1-13.

Stebeck, C. E., Baron, G. S., Beecroft, R. P. and Pearson, T. W. 1996. The molecular characterization of the kinetoplastid membrane protein-11 from African trypanosomes. Molecular and Biochemical Parasitology, in press.

GENERAL DISCUSSION

The original objective of my thesis research was to identify and characterize invariant molecules present on the cell surface of the procyclic life cycle stage of African trypanosomes. Although this objective was not accomplished, my research led to the identification and characterization of two invariant membrane-associated molecules from procyclic trypanosomes. The first of these molecules, the NAD^+-dependent glycerol 3-phosphate dehydrogenase, is localized within the glycosome. Its apparent association with the trypanosome plasma membrane remains a mystery. The second molecule identified and characterized, the trypanosome kinetoplastid membrane protein-11, forms an amphipathic helical structure and in this way could clearly associate with the cell membrane, although the precise membrane disposition of this molecule is still unresolved.

The uncertainty surrounding the cell surface locale of these proteins in no way diminishes their importance. The NAD^+-dependent glycerol 3-phosphate dehydrogenase plays a pivotal role in the glycolytic pathway and therefore represents an essential enzyme to bloodstream trypanosomes which rely on glycolysis for derivation of energy. The trypanosome enzyme shares only 23.5% sequence identity with the corresponding enzyme from humans and exhibits minimal conservation of predicted active site residues with its mammalian counterpart. It is thus thought to represent an ideal target for design of trypanocidal chemotherapeutic agents. The kinetoplastid membrane protein-11 is widely distributed throughout kinetoplastid parasites and therefore may be functionally essential to these protozoans. Its role in kinetoplastids remains unknown.

Results presented in this thesis have demonstrated that the *T.b. rhodesiense* kinetoplastid membrane protein-11 and NAD^+-dependent glycerol 3-phosphate dehydrogenase are encoded by low copy number genes (one and two gene copies, respectively), and hence these membrane-associated molecules are ideal candidates for knockout mutagenesis to ascertain their functional relevance to African trypanosomes.

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