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Membrane glycoconjugates of procyclic *Trypanosoma simiae* and *Trypanosoma congolense*, members of the subgenus *Nannomonas* are immunologically similar and biochemically distinct.

By

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B.Sc., University of Bombay, 1989
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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

In the Department of Biochemistry and Microbiology

We accept this dissertation as conforming to the required standard

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Abstract

The surface molecules (procyclins) of procyclic forms of African trypanosomes (*Trypanosoma brucei* spp.) are complex mixtures of lipid-anchored glycoconjugates. The procyclins are expressed differentially during the parasite life cycle within the tsetse fly vector. It has been hypothesised that these surface molecules are involved in interactions with molecules of the tsetse fly and may influence differentiation, cell death and tissue tropism. To understand procyclin functions it is necessary to identify and characterise them. This thesis presents a study of the biochemical and immunochemical characteristics of the major surface molecules of *Trypanosoma simiae* and *Trypanosoma congolense*, animal pathogens of the subgenus *Nannomonas* that share the same developmental cycle and tropism within the tsetse vector.

Organic solvent extraction, reverse-phase high performance liquid chromatography and enzyme-linked immunosorbent assay using surface binding monoclonal antibodies were used to isolate membrane molecules of procyclic culture forms (PCF) of both trypanosome species. Gel electrophoresis of the purified molecules revealed two predominant molecular species from each parasite that were broadly similar yet showed different apparent molecular masses and staining characteristics. The molecules were shown to be glycosylphosphatidylinositol-lipid anchored glycoconjugates, comprised mainly of carbohydrates. Each moiety displayed surface - disposed carbohydrate epitopes that were recognised on the surface of both species of trypanosomes by monoclonal antibodies specific for procyclic parasites of the subgenus *Nannomonas*. The epitopes were previously shown to be displayed on the glutamic acid-alanine rich protein (GARP) of *T. congolense*, yet neither this protein (as detected either

immunologically or by mass spectrometry) nor its encoding gene (as detected by Southern blot analysis) was present in *T. simiae*. The results indicate that although *T. congolense* and *T. simiae* share common carbohydrate surface epitopes, these are displayed on biochemically different molecules. I hypothesise that the surface disposed carbohydrate structures and not the polypeptide moieties are involved in parasite-tsetse interactions since these species have the same developmental cycles in the insect vector.

In an attempt to obtain primary sequence information for the *T. simiae* PCF surface molecules, I identified and characterised an unique open reading frame. This was shown to be expressed as a protein in PCF and is likely a membrane-associated molecule of the subgenus *Nannomonas*.

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Abbreviations used

VSG, variant surface glycoprotein.

BSF, bloodstream forms.

PCF, procyclic culture forms.

GPI, glycosylphosphatidylinositol.

GPI-PLC, glycosylphosphatidylinositol - phospholipase C.

MfVSG, membrane form of VSG.

ES, expression sites.

ESAGs, expression site-associated genes.

PAGs, procyclin-associated genes.

GRESAG, genes related to ESAG.

MEM, minimum essential medium.

FBS, foetal bovine serum.

MAb, monoclonal antibody.

SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

PVDF, polyvinylidene difluoride.

ELISA, enzyme-linked immunosorbent assay.

TFMSA, trifluoromethane sulfonic acid.

HPLC, high performance liquid chromatography.

MALDI-TOF, matrix-assisted laser desorption ionization time-of flight.

ESI, electrospray ionization.

ESI-QTOF, electrospray ionization (nanospray)-quadrupole time-of-flight.

CNBr, cyanogen bromide.

PCR, polymerase chain reaction.

ORF, open reading frame.

KMP –11, kinetoplastid membrane protein – 11.

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1. INTRODUCTION

In the 21st century where yesterday's science fiction often becomes reality, our "developed" world has yet to conquer the infamous African trypanosomes. The destruction caused by these parasites has been documented since the 1850's. However, it was only at the beginning of the 20th century that the causative agent and link to its mode of transmission were identified. In the past few years, socio-economic and political instability in the African continent has led to the unfortunate re-emergence of epidemics due to trypanosomiasis, with major incidences occurring in Angola, Zaire, Uganda, Democratic republic of Congo and Sudan, resulting in more than 100,000 new infections reported per year [Welburn *et al.*, 2001]. According to the World Health Organization (WHO), in 1995 there were an estimated 55 million people at risk of sleeping sickness in Africa, with 300,000 to 500,000 cases occurring per annum [Welburn *et al.*, 2001; Hide G, 1999; Smith *et al.*, 1998]. If potential losses in livestock and crop production are taken into consideration, then trypanosomiasis may cost Africa more than US $ 5 billion per year (ILRAD, 1994). Health care control programs have fallen apart today as many of the affected countries are wrecked by post-colonial political struggle disrupted by civil wars. My sentiment during my Ph.D. program was to be a part (however miniscule it might be) of the community trying to restrain the devastation of the so-called "third world" by infectious diseases. It is even more evident to me today that co-operative global mobilization of effort (monetary, humanitarian and intellectual) is essential before anyone can begin to track and control the havoc created by a variety of infectious diseases in Africa, important among these being the trypanosomes in both humans and livestock.

African trypanosomes are responsible for trypanosomiasis, often a fatal disease called Nagana in livestock and sleeping sickness in humans. African sleeping sickness is characterised by two distinct phases: an early phase with symptoms being nausea, lethargy and fever and a late phase during which the trypanosomes cross the blood-brain barrier and are consequently found in neural tissue and cerebrospinal fluid. The symptoms of the late phase explain the metaphorical name "sleeping sickness" that results in disruption of biological rhythms, inappropriate and irregular sleeping patterns and loss of concentration and coordination. The late phase of sleeping sickness is usually fatal unless treated by administration of toxic trypanocidal drugs. Unfortunately, the drugs themselves can cause fatality in up to 5% of patients!

African trypanosomes are pathogenic, unicellular, eukaryotic, tsetse-transmitted protozoan parasites of the genus *Trypanosoma* (refer to figure 1.1 for a diagrammatic representation of the structure of these parasites). *Trypanosoma brucei rhodesiense* is the causative agent of the acute form of human African sleeping sickness in East Africa, which is characterised by rapid progression to meningoencephalitis. The causative agent of the chronic form of human African sleeping sickness, with an initial long asymptomatic stage and late meningoencephilitis, is *T. b. gambiense*, which exists in west and central Africa. Human sleeping sickness caused by *T. b. gambiense* is often endemic at high levels and is largely undocumented during transmission as the early symptoms are mild, whereas the disease caused by *T. b. rhodesiense* is acute and epidemic. It is interesting how these two species of *T. brucei*, though morphologically indistinguishable, have evolved to be specific in terms of clinical presentation and geographical location, with a clear boundary delineated by the north-south orientation of the Great Rift Valley.

Geographically, Uganda is the meeting point of the two species, with *T. b. rhodesiense* in the northwest and *T. b. gambiense* in the southeast. The third species of the subgenus *Trypanozoon, T. b. brucei*, is non-infective to humans and infects a variety of domestic animals including economically important cattle, across sub-Saharan Africa.

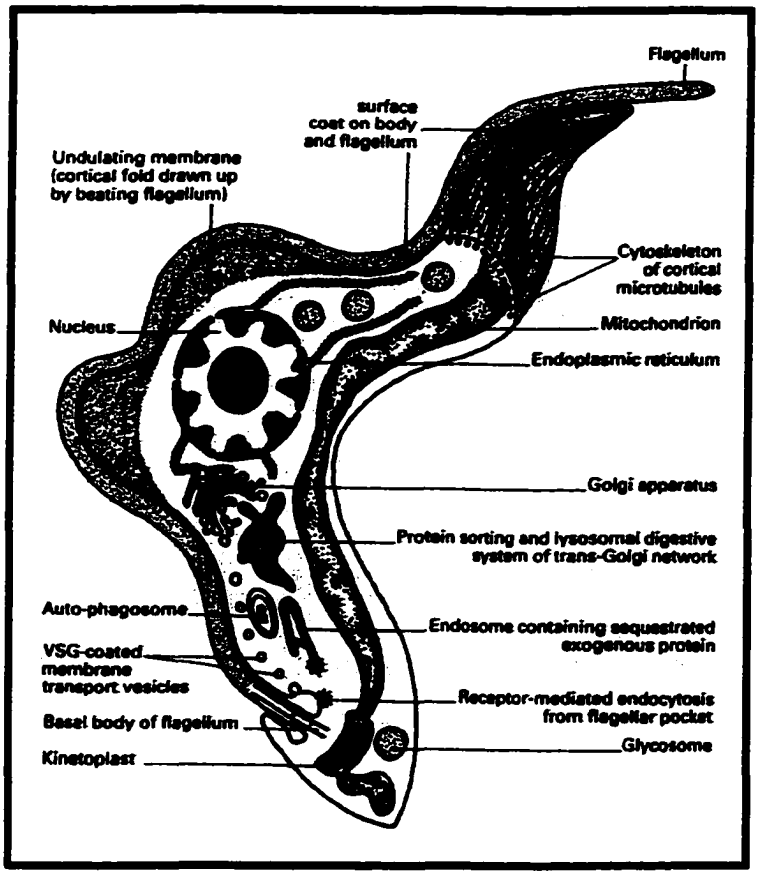


Figure 1.1. ***Diagrammatic representation of the structure of T. brucei [Vickerman et al., 1993]***

African trypanosomes also infect a wide spectrum of wild animals which act as reservoirs for parasites that infect domestic livestock. Along with *T. brucei,* members of the subgenus *Nannomonas* (*T. congolense*) and subgenus *Duttonella* (*T. vivax*) cause trypanosomiasis in cattle and can also infect other economically important domestic

animals such as sheep and goats. Another member of the subgenus *Nannomonas*, *T. simiae*, causes acute trypanosomiasis in pigs and less virulent infections in sheep, goat, rabbits, certain primates and other exotic animals, such as the white rhinoceros and camels [Mihok *et al.*, 1994], with wart-hog and bushpigs serving as reservoirs. Examples of other animal-infective African trypanosomes are the sexually - transmitted equine parasite *T. equiperdum*, the camel parasite, *T. evansi* and the crocodile parasite *T. grayi*. Because of the wide host range of trypanosomes, it is a difficult task to isolate and identify different species of these parasites, leading to the suggestion that there maybe other "elusive" species not yet detected in the wild [Dukes *et al.*, 1991].

1.1. *African trypanosomes: an ancient existence*

African trypanosomes belong to the order Kinetoplastida, the name associated with the presence of a large, dense organelle containing mitochondrial DNA found at one end of the base of the flagellum, the kinetoplast. Kinetoplast DNA contains 20-50 copies of a 22 kb maxicircle and up to 100,000 minicircles of 1 kb each. All kinetoplastids have two other major characteristic features: compartmentalization of glycolysis within a microbody called the glycosome and trans-splicing of a highly conserved 39 nt RNA leader transcript called the mini-exon (the function of which is yet to be determined) onto every pre-mRNA to generate mature mRNA. Transcription of protein-coding genes and mRNA maturation exhibited by these parasites is thus distinct from other eukaryotes in that the 5' end of all mature mRNA in trypanosomes is formed post-transcriptionally.

The 5' mini-exon transcript is added by trans-splicing of the mini-exon during pre-mRNA maturation [Boothryod and Cross, 1982]. Recently cis-splicing of RNA has also been described in trypanosomes [Tschudi and Ullu, 2000]. Another unique molecular mechanism exhibited by these parasites is RNA polymerase I - mediated expression of mature mRNA for some protein coding genes, such as the major variant surface glycoprotein (VSG) in the mammalian host and the procyclins in the arthropod vector [for review see Lee and Van der Ploeg, 1997].

Phylogenetic studies using ribosomal RNA (rRNA) coupled with morphological evidence, suggests that kinetoplastids have a single evolutionary lineage [Wright *et al.*, 1999]. African trypanosomes belong to the suborder Trypanosomatina (kinetoplastida with one flagellum) consisting of a single family, Trypanosomatidae. Members of this family are all obligate parasites. Further, the African trypanosomes belong to the genus *Trypanosoma*. The defining characteristic of this genus is that all the members are digenetic, with the life cycle being cyclical, alternating between vertebrate hosts and the gut of arthropods. Trypanosomes are believed to have diverged as a monophyletic clade from other kinetoplastids around 400 – 600 million years ago [Overath *et al.*, 2001]. There are two major divisions of the genus *Trypanosoma*: *Stercoraria* and *Salivaria*, depending on their course of development in the vector. *Stercorarian* trypanosomes develop in the terminal gut of the arthropod vector and transmission is contaminative through the vector faeces. In contrast, *salivarian* trypanosomes develop in the anterior gut of the arthropod vector and transmission is inoculative via the saliva of the vector. *Salivarian* trypanosomes are estimated to have diverged from non-*salivarian* trypanosomes between 200 and 300 million years ago [Overath *et al.*, 2001]. Thus the

salivarian trypanosomes has been estimated to have originated before the appearance of placental mammals (which appeared < 100 million years ago) or the vector of our interest, the tsetse fly (estimated to have appeared 30 – 60 million years ago), or even before the separation of the African continent from South America, estimated to be 80 – 100 million years ago [Overath *et al.*, 2001]. All *salivarian* trypanosomes evade the immune response of their hosts by expression of antigenically distinct surface molecules by differential activation of previously inactive *vsg* genes (see section 1.3 for a detailed discussion of VSG and antigenic variation). This sophisticated mechanism of survival may be due to the fact that the African trypanosomes have shared ~15 million years of co-evolution with primates [Stevens *et al.*, 2001].

1.2. *African trypanosomes: two high maintenance life styles*

The life cycle of African trypananosomes is complex as the parasites alternate between a mammalian host and an arthropod vector, the tsetse fly (*Glossina* spp.), during which they undergo both proliferative and non-dividing phases [for reviews see Mulligan, 1970; Shapiro and Pearson, 1986; Vickerman *et al.*, 1988]. The two major morphological forms of trypanosomes are trypomastigotes (kinetoplast posterior to the nucleus) in the bloodstream of the vertebrate host and epimastigotes (kinetoplast adjacent to and anterior to the nucleus) in the arthropod vector. Due to the drastic environmental changes in the two hosts, the trypanosomes respond with equally extreme adaptive changes during the different stages of their life cycle. These include a complete change of their surface coat during transition from the mammalian forms to the insect forms of the parasite.

The life cycle of African trypanosomes has been most extensively studied in *T. brucei* spp. (Figure 1.2).

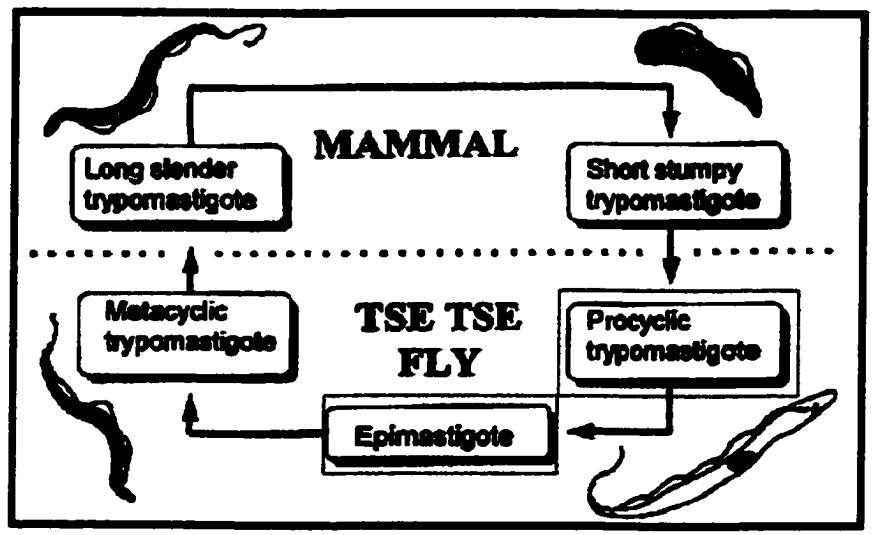


Figure 1.2. ***Life cycle of African trypanosomes alternating between two hosts.***

The dark colour represents the variant surface glycoprotein coat. Procyclic and epimastigote forms have a procyclin coat, represented by the red colour.

a) ***Life cycle of trypanosomes in the mammalian host***

Metacyclic trypomastigotes, the mammal - infective form, are injected into the dermal connective tissue of the mammal via the tsetse vector's saliva. During the initial stages, the parasites localise within the dermis at the bite site and then rapidly differentiate into proliferative long, slender bloodstream forms (BSF). These BSF parasites subsequently escape the bite site, enter the draining lymphatics and the bloodstream of the host (systemic stage). The BSF can ultimately invade the central nervous system during the advanced stages and are found in neural tissue and cerebrospinal fluid. BSF trypanosomes are adapted to exploit the glucose rich environment in the mammalian blood and have repressed mitochondria that lack cristae

[Vickerman *et al.*, 1988]. These BSF parasites depend entirely on glycolysis for ATP generation.

BSF trypanosomes have developed an extremely sophisticated tactic, antigenic variation, to escape the immune response of the host. The major surface glycoprotein, VSG, expressed on the surface of BSF, is expressed as a new antigenic variant at each successive wave of parasitemia, thus evading the host's immune response (explained in detail in section 1.3) [Vickerman *et al.*, 1985]. BSF parasites rapidly proliferate as long, slender forms and when they reach a specific threshold density, "short stumpy" BSF begin to appear [Seed and Black, 1997]. This transformation appears to be an early adaptation mechanism for life in the tsetse fly vector. The short stumpy BSF have a semi-developed mitochondrion as they activate some components that are required for a functional mitochondrion in the tsetse fly. Tubular cristae develop and proline oxidase and oxoglutarate oxidase systems appear for use in the tsetse fly [Vickerman *et al.*, 1988]. Though kinetoplast maxicircle genes are transcribed in all three proliferative major stages of the life cycle (long slender BSF, short stumpy BSF and procyclic forms), the abundance of specific transcripts varies depending on the developmental control of mitochondrial activities. Mitochondrial ribosomal RNA levels are 30-fold lower in slender BSF than in stumpy BSF and further, the transcripts in stumpy forms are not translated until transformation to procyclic forms takes place. Short stumpy forms are also thought to express simultaneously with VSG, low levels of procyclins, the immunodominant surface coat in the insect forms of the trypanosomes. Interestingly, fly - infective short stumpy BSF are non-dividing and are eliminated from the host unless they are taken up by the tsetse fly. This phenomenon appears to maintain persistent

infection in the mammalian host and increases the probability of completion of the parasite life cycle. Not all long slender BSF differentiate into short stumpy forms. A few parasites still proliferate as long - slender forms even after the majority of the population has switched to short stumpy BSF. Bloodstream trypanosomes also have the capacity to take up specific plasma proteins by receptor-mediated endocytosis through the flagellar pocket whereas this ability is lost in the insect forms of the parasites.

b) ***Life cycle of trypanosomes in the tsetse vector***

Trypanosomes ingested by the tsetse fly in a bloodmeal lose infectivity for mammalian hosts within 24 hr and transform to insect midgut-adapted forms, the procyclic trypomastigotes, within a period of 2-3 days [Vickerman *et al.*, 1985]. Procyclic forms have a fully functional mitochondrion and exhibit oxidative phosphorylation for generation of ATP using proline as the main energy source. These parasites, adapted to survive in the fly midgut, do not express VSG. However, they express a new set of immunodominant, GPI-anchored, surface glycoproteins, the procyclins (discussed in detail in 1.4).

Once an infection is established in the tsetse fly, a process called establishment, the procyclic forms penetrate the peritrophic membrane and remain at a fairly constant population density in the ectoperitrophic spaces. It is thought that the population density is maintained by balancing parasite multiplication with programmed cell death [Welburn and Maudlin, 1999; Pearson *et al.*, 2000]. These parasites retraverse the peritrophic membrane and migrate forward to the proventriculus, where they cease to divide and elongate to form mesocyclic trypomastigotes. The differentiation pathway of

trypanosomes in the tsetse vector differs here, depending on the subgenus. Members of the subgenus *Trypanozoon* make their way to the salivary glands of the vector where they anchor to the epithelium microvilli. Here they differentiate into epimastigote forms, which in turn differentiate into non-dividing, mammal-infective metacyclic forms that acquire a VSG coat. This process is called maturation. In contrast, epimastigotes of members of the subgenus *Nannomonas* multiply while attached to the chitinous wall of the proboscis and the premetacyclics migrate to the hypopharynx where they mature into infective metacyclic forms. Inhibition of attachment of the epimastigotes does not appear to prevent division of the parasites, however, this does inhibit differentiation into metacyclic forms implying that the attachment of the epimastigotes seem to have a developmental significance [Vickerman *et al.*, 1988]. The paraflagellar rod has been hypothesized to play a role in the attachment of the parasite via the flagellum [Thévenaz and Hecker, 1980; Gull K, 1999]. Metacyclic forms, like short stumpy BSF, are non-dividing and like all BSF are adapted to survive in the mammalian bloodstream and express VSG. The entire developmental cycle in susceptible tsetse flies takes 3-5 weeks. Interaction of midgut lectins in the tsetse fly with parasite surface molecules, by inference, with carbohydrates, has been postulated to play a central role in differentiation of trypanosomes in the fly [Maudlin and Welburn, 1988] and may also play a role in apoptosis of the parasites [Pearson *et al.*, 2000]. However, no tsetse lectins have been biochemically characterised and the role of the tsetse molecules in parasite differentiation or death has not been elucidated.

Although most species of African trypanosomes are entirely dependent on tsetse for their transmission, many flies are not easily infected. Susceptibility to midgut

infections has been linked to the presence of maternally inherited bacterial symbionts and the efficiency of transmission has been linked to the species and the sex of the tsetse fly [Maudlin and Welburn, 1994; Welburn and Maudlin, 1999]. In the tsetse vector, the parasites not only have to establish an actively dividing procyclic population in the midgut (establishment), but also have to retraverse the peritrophic membrane, leave the midgut and migrate to the appropriate compartment to differentiate into mammal-infective metacyclic forms (maturation). The importance of cyclical transmission of trypanosomes by tsetse is evident from the overlapping distribution pattern of trypanosomiasis and tsetse flies in sub-Saharan Africa (Figure 1.3).

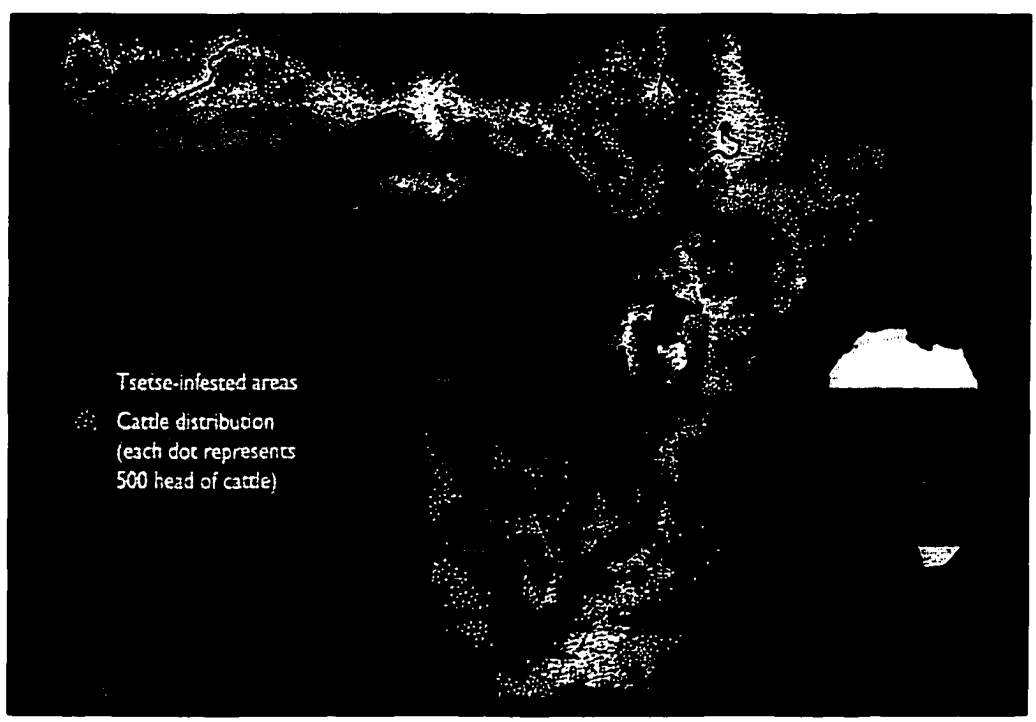


Figure 1.3. ***Overlapping distribution of tsetse-infested areas with trypanosomiasis in cattle.***

The African trypanosome has been extensively studied from a molecular approach. Most research has involved the surface coat molecules of BSF and more recently, of the procyclic culture forms (PCF), because of their perceived importance for both host-parasite and vector-parasite interactions respectively.

1.3. *Bloodstream forms: the surface coat*

a) *Variant surface glycoproteins*

In the mammalian host, African trypanosomes express a 12-15 nm thick, immunodominant surface coat comprised of the variant surface glycoprotein (VSG) [Boothroyd, 1985; Cross, 1990; Vickerman *et al.*, 1993; Pays and Nolan, 1998]. VSG accounts for about 10 % of the total cell protein in BSF (10^7 molecules / cell) and is expressed throughout the life cycle of the parasite in the host mammal. The VSG coat protects the parasite against complement-mediated lysis in a non-immune mammalian host and, as a population, against host antibodies in an immune animal (or at least one making an immune response). VSG is expressed as a glycosylphosphatidylinositol (GPI) - anchored glycoprotein containing myristic acid residues as the lipid anchor, with a variable N-terminal domain of 300 – 400 amino acids and a relatively conserved C-terminal domain of 50 – 100 amino acids. Expressed VSG molecules are N-glycosylated and are assembled as dimers. The N-terminal domains fold into two anti-parallel ∝-helices resulting in an extended structure perpendicular to the cell surface [Blum *et al.*, 1993]. It is interesting that different VSGs, with different amino acid sequences and no immunological cross-reactivity, have highly conserved three-dimensional structures. The structural organisation of the molecules is such that the conserved C-terminal amino acids

are not exposed and only a limited stretch of variable amino acids (of the N-terminal domain) are accessible to the extracellular environment [Blum *et al.*, 1993]. This structural organisation is thought to prevent immune recognition of the invariant epitopes of the C- terminal region. There are two classes of VSG: Class I, where aspartic acid or asparagine is the C-terminal amino acid to which the GPI anchor is attached and Class II, where the C-terminal amino acid bound to the lipid anchor is serine.

The *T. brucei* genome is estimated to contain as many as 1000 different *vsg* genes [Van der Ploeg *et al.*, 1982], which implies that more than 2% of the trypanosome genome is devoted to the coding of VSG. Only one *vsg* gene (and protein) is expressed at a time and the expressed protein represents the membrane form of VSG (mfVSG). The mfVSG is responsible for the differences between the serologic variants or the variant antigen type (VAT) of BSF trypanosomes. Antibodies elicited to the mfVSG by the host react only with parasites of the same VAT. This results in the rapid clearing of the parasites. However, a few parasites in the population will express a new mfVSG molecule on their surface and subsequently escape the humoral response of the host to the previous mfVSG. These survivors quickly proliferate and present the immune system of the host with a new mfVSG molecule. The trypanosomes thus make use of the host's immune system to control their growth and maintain a constant and tolerable number of the parasites in the bloodstream. Thereby, a persistent infection in the mammalian host is established. Trypanosomes can sequentially express more than one hundred and possibly thousands of different mfVSGs. It is thought that a trypanosome GPI-associated phospholipase C (GPI-PLC) cleaves the GPI anchor releasing the soluble form of mfVSG (which retains only the glycosylphosphatidylinositol phosphate, VSG-GIP), leaving the

dimyristoylglycerol lipid component in the membrane. This released soluble form of VSG-GIP results in activation of host macrophages [Paulnock and Coller, 2001]. Although the component of the released VSG-GIP that interacts with the macrophage membrane has not been identified, it is thought to be a specific receptor-mediated interaction. The interaction of VSG-GIP with the host macrophage is postulated to contribute to a polarization toward a Th1 immune response in the host with elevated IL-12, IL-6 and TNF-∝ (trypanolytic cytokine) levels [Paulnock and Coller, 2001].

Transcription of *vsg* genes occurs in *vsg* expression sites (ES) that are polycistronic units in the BSF. The expressed *vsg* gene is always located adjacent to a telomere, 1 – 3 Kbp upstream of a TTAGGG hexameric telomeric repeat. The telomeric *vsg* genes are flanked by upstream 70 bp repeats (the remnants of which are found in some non-telomeric *vsg* genes, thought to be originally translocated from telomeric ends) and downstream by polymorphic telomeres. The characteristic features of the ESs are that they are at the telomeric ends of chromosomes and that they have additional genes known as expression site-associated genes (ESAGs) with varying order and copy number between ES's. ESAGs belong to large gene families and not all of them are restricted to the expression of VSG [Pays *et al.*, 2001]. Some characteristics of the known ESAGs and the proteins encoded by them are shown in Table 1.1.

Table 1.1. ***Characteristics of expression site associated genes (ESAGs) and their products [modified from Pays et al., 2001].***

ESAG *genes / encoded proteins*	Similarity to known existing sequences	Localization in the trypanosome	Description or function (postulated)
ESAG 1	-	Flagellar pocket	Receptor (?)
ESAG 2	-	Flagellar pocket	GPI-anchored glycoprotein
ESAG 3	-	Secreted (?)	Surface glycoprotein
ESAG 4	Adenylyl cyclase catalytic domain	Flagellar surface	Receptor-like adenylate cyclase
ESAG 5	-	?	?
ESAG 6	VSG	Flagellar pocket	Transferrin receptor subunit
ESAG 7	VSG	Flagellar pocket	Transferrin receptor subunit
ESAG 8	Ring finger proteins	Nucleus	Control factor (?)
ESAG 9	GTP binding proteins	Surface membrane	GPI-anchored glycoprotein
ESAG 10	Pteridine transporter	Integral membrane	Biopterin transporter
ESAG 11	-	Surface membrane	GPI-anchored glycoprotein

There are two sets of ESs, one in BSF and the other in metacyclic forms (in the tsetse fly). BSF ES's are polycistronic units with ESAGs and arrays of 70 bp repeat between the *vsg* genes and ESAGs and they are transcribed by a promoter 30 – 50 kb upstream of the telomere. In contrast, the metacyclic ESs are monocistronic, being transcribed by a promoter immediately upstream of the *vsg* gene and they do not contain functional ESAGs or the 70 bp repeats. Though the main reservoirs of *vsg* genes are the telomeric ends of the mini-chromosomes, the *vsg* genes in the mini-chromosomes cannot be transcribed *in situ* as they are not preceded by a transcription promoter. It is only the telomeric loci of the large chromosomes (the ESs) that have the potential to be transcribed *in situ*. Of the 20 known *vsg* ESs in BSF, only one ES is active at any given

time and of the 1000 *vsg* genes only one is expressed at any given time. This phenomenal regulation evolved by the African trypanosomes is the crux of their survival in mammals.

The process of antigenic variation takes place either by DNA recombination which allows silent *vsg* cassettes to be shuttled into an active ES by the process of duplicative transposition (similar to gene conversion) or by a less specific recombination event of duplicative transposition of large silent telomeric regions or ESAGs into an active ES [for reviews of antigenic variation see Borst *et al.*, 1998; Cross *et al.*, 1998; Rudenko, 2000; Pays *et al.*, 2001; Vanhamme *et al.*, 2001]. The flanking 70 bp repeats at the ESs is not only thought to facilitate the DNA recombination events but also may be the target for nuclease activity that initiates duplicative transposition of a *vsg* gene into an active ES. Antigenic variation can also take place by transcriptional switching of *vsg* genes between ESs by simultaneous activation of a new ES and silencing of an active ES. Regulation of VSG expression has been extensively studied [Borst *et al.*, 1998; Cross *et al.*, 1998; Vanhamme and Pays, 1998; Vanhamme *et al.*, 2000; Vanhamme *et al.*, 2001]. It appears that the transcription promoters of both stage-specific major surface glycoproteins in these parasites, VSG and procyclins, are active during all of the stages of the parasite life cycle. Also, transcription can be initiated from multiple ES's in BSFs. Thus the regulation of VSG expression is primarily controlled at the level of RNA elongation, RNA processing, nuclear transport and RNA stabilization, which preferentially takes place only at the active ES [Vanhamme *et al.*, 2000; Vanhamme *et al.*, 2001]. A number of events regulate *vsg* genes during differentiation of BSF to procyclic forms. Once differentiation is induced, the nascent transcripts from the *vsg* ES

are decreased and RNA elongation is stimulated in the procyclin transcriptional units. Hence it is RNA elongation that is regulated positively in a stage specific manner and VSG and procyclin mRNA levels are regulated inversely during the differentiation process [Roditi *et al.*, 1989]. It also has been postulated that the 3' UTR of the *vsg* mRNA stabilizes the mRNA through interaction with certain unidentified labile protein factors that maybe involved in stabilization of some ESAG mRNA. These factors are postulated to be lost on differentiation of the BSF to procyclic forms resulting in destabilisation of the mRNAs [Vanhamme and Pays, 1998].

The complex transcription pattern of *vsg* gene from an active ES and the efficient processing of the subsequent ES transcript is not clearly understood. The fact that *vsg* gene transcription is RNA polymerase I mediated, yet processing of the transcript is by the eukaryotic mRNA machinery of splicing and polyadenylation, leads to the belief that there has to be a special regulatory system in these parasites linking polymerase I to eukaryotic mRNA processing [Vanhamme, 1998; Vanhamme, 2001; Pays *et al.*, 2001].

b) ***Other surface molecules expressed in BSF trypanosomes***

For survival and multiplication in the host it is evident that African trypanosomes would also require other surface receptors for uptake of nutrients and for transducing signals to regulate their various metabolic pathways. Non-VSG surface molecules have also been characterised from the BSF of the parasites. Several of these molecules are associated with the plasma membrane of the flagellar pocket. These invariant surface receptors have been associated with substrate uptake [Borst and Fairlamb, 1998]. How the BSF of the parasites combine antigenic variation with the expression of these

invariant surface molecules that function as receptor molecules is unknown. However, it is clear that these receptors are hidden in the flagellar pocket from macrophages and T-cells. Also, one of these molecules, the tranferrin receptor, is known to show some antigenic variation, which helps to minimise the antibody response. In addition, the high rate of endocytosis of the flagellar pocket membrane has been postulated to continuously deplete the flagellar pocket of antibodies [Borst and Fairlamb, 1998]. Some of the known *T. brucei* surface-associated receptor molecules and their characteristics are listed in Table 1.2.

Table 1.2. ***Surface receptors of BSF of T. brucei.***

Surface-associated receptor molecules	Characteristics	Postulated functions	References
Transferrin Receptor	Expressed only in BSF. Two subunits, encoded by ESAG6 and ESAG 7. BSF express approximately 20 different transferrin receptor molecules.	Uptake of transferrin, which is the only known source of iron in BSF and an essential growth factor for BSF.	Coppens *et al.*, 1987. Steverding *et al.*, 1994.
Adenylate cyclases	Encoded by ESAG4. Basal adenylate cyclase activity is stimulated by Ca^{+2} in BSF but not in procyclic forms. *T. brucei* cyclase has a conserved intracellular catalytic domain, a single transmembrane domain and a large less conserved extracellular N-terminal domain.	The extracellular domain is responsible for receptor function and is thought to be involved in signal transduction.	Pays *et al.*, 1989. Alexandre *et al.*, 1996.
Low-density lipoprotein (LDL) receptor	Cysteine-rich, acidic membrane protein (?)	Receptor-mediated endocytosis of LDL from mammalian host.	Coppens *et al.*, 1988. Lee *et al.*, 1990. Coppens *et al.*, 1991.
Invariant surface glycoproteins (ISG)	They are estimated to be present at about 0.5% of the copy number of VSG. Two of the ISGs, 65 and 70, are expressed over the entire surface of *T. brucei* (not just restricted to the flagellar pocket) and both of these ISG have N-terminal glycosylated extracellular domains. All of the known ISG lack homology with known proteins.	The functions of these ISG are largely unknown, they are thought to act as receptors for small ligands and not macromolecules.	Ziegelbauer *et al.*, 1992. Ziegelbauer *et al.*, 1993. Jackson *et al.*, 1993. Overath *et al.*, 1994. Ziegelbauer *et al.*, 1995.
Putative receptor for Epidermal growth factor (EGF).	A homologue of the mammalian EGF receptor.	The putative receptor has not been purified nor the gene cloned and its function remains unknown.	Hide *et al.*, 1989.
Putative High-density lipoprotein (HDL) receptor.	Cysteine-rich acidic integral membrane protein (CRAM), mRNA detected BSF and procyclic forms.	Postulated to be receptor for HDL.	Lee *et al.*, 1990.

1.4. *Insect forms: the surface coat*

a) *Procyclins - a coat of many colours*

The most dramatic event during the transformation of BSF to procyclic forms is the loss of the VSG coat and the appearance of a new surface coat comprised of various forms of procyclins. The synthesis of procyclin mRNA and expression of the procyclin proteins are the earliest known markers of this differentiation event and occur even before the loss of the VSG coat [Roditi *et al.*, 1989]. During the BSF - PCF transformation, intermediate forms of trypanosomes can be detected that simultaneously express both VSG and procyclin [Roditi *et al.*, 1989]. To explain this, it has been hypothesised that the procyclin molecules have an extended structure that projects out through the VSG coat. Later, the VSG is replaced by procyclin on the surface of the parasites and as a result, at no time are these parasites uncoated [Roditi and Pearson, 1990]. A lag in shedding of VSG is advantageous for survival of the parasites during adaptation from the mammalian host to the tsetse fly vector, since premature loss of VSG or early induction of procyclin expression could be lethal to the parasite in the mammalian bloodstream. It is interesting that the GPI-PLC enzyme possessed by trypanosomes is not responsible for the shedding of the VSG coat. Deletion of the GPI-PLC genes from the trypanosome genome does not affect VSG switching or VSG loss during differentiation and growth alteration is seen only in null mutants in the rodent host [Webb *et al.*, 1997]. It has been suggested that a metalloprotease is responsible for the shedding of the VSG coat [Bangs *et al.*, 1997]. During differentiation of the BSF, procyclin mRNA is detected early, within 2 hr after the start of transformation, preceding any morphological changes in the BSF. In spite of the rapid mRNA detection there is a lag in protein expression, as the procyclin protein is not detected until 8 hr after induction

of differentiation, reaching a maximum level of expression within 24 hr [Roditi *et al.*, 1989]. Post transcriptional control with the requirement of an additional signal for translation of procyclin mRNA seems to be necessary for protein expression.

Transformation of BSF to PCF can be induced *in vitro* by reducing the temperature from 37 °C to 27 °C and by addition of some of the Krebs cycle intermediates (citrate and cis-aconitate). Cis-aconitate plays a central role in induction of differentiation *in vitro* coupled with reduction of temperature without any deleterious effect on cell viability and can trigger differentiation of both long slender and short stumpy BSF [Roditi *et al.*, 1989; Mathew *et al.*, 1999; Sbicego *et al.*, 1999]. However, as cis-aconitate is effective only in relatively higher concentrations *in vitro*, it is unlikely that this has any physiological implications. Differentiation *in vitro* can also be induced by treatment of the cells with proteases such as pronase [Hunt *et al.*, 1994], trypsin [Yabu, 1993] and thermolysin [Sbicego *et al.*, 1999]. It is interesting to note that both trypsin and thermolysin selectively kill > 90 % of the long slender BSF during induction of differentiation *in vitro* within 15 min of incubation, whereas the majority of the short stumpy BSF are resistant to the proteases [Sbicego *et al.*, 1999]. A combination of trypsin treatment with cis-aconitate results in enhancing the kinetics of differentiation of stumpy BSF with expression of procyclin within 3 hr, which is similar to the expression kinetics seen with trypsin treatment alone [Sbicego *et al.*, 1999]. Therefore it appears that it is the protease-induced transformation that has relevance in the fly and that midgut proteases play a central role [Hunt *et al.*, 1994]. When differentiation is induced *in vitro*, the long slender BSF of trypanosomes do not transform in synchrony and there is a lag of 24- 30 hr for some of the population, as they must reach a certain position in the life

cycle to be competent to differentiate [Roditi *et al.*, 1989]. In contrast, short stumpy BSF differentiate synchronously once induced, express procyclin within 2 hr and lose VSG within 4 hr [Roditi *et al.*, 1989; Ziegelbaur *et al.*, 1990; Vassella *et al.*, 1997].

The procyclins are a set of heterogeneous, stage specific, acidic, immunodominant, GPI-anchored surface glycoproteins that are expressed in the procyclic and epimastigote forms of African trypanosomes. The name "procyclin" was initially given to one form (the EP- form) of procyclin in *T. brucei* because of its stage-specificity and high proline content [Richardson *et al.*, 1988]. Approximately 2 X 10^6 copies of procyclin molecules are expressed per cell in the *T. brucei* clones so far analysed.

Procyclins in *T. brucei* are encoded by a small number of genes and are transcribed as a polycistronic unit. All genes are contained within four expression sites per diploid genome (Figure 1.4). Two major forms of procyclin are expressed in *T. brucei*: the EP-procyclins and GPEET-procyclins, that differ in their repeat sequences and in post-translational modifications. In addition, the EP- and GPEET- procyclins express several isoforms for which a formal nomenclature has been proposed [Roditi and Clayton, 1999]. Three genes designated as *EP1, EP2* and *EP3* encode the various isoforms of EP-procyclins] and a fourth gene, *GPEET*, encode GPEET procyclins [Ruepp *et al.*, 1997; Roditi *et al.*, 1998; Roditi and Clayton, 1999]. Each is present in two copies per diploid genome. Downstream of the procyclin genes in each expression site are procyclin-associated genes (PAGs) and downstream of the GPEET - PAGs are the genes related to ESAG (GRESAG). Similar to *vsg*, procyclin genes are transcribed by the α-amanitin-resistant, RNA polymerase I-like polymerase [Lee and Van der Ploeg, 1997; Roditi *et al.*, 1998].

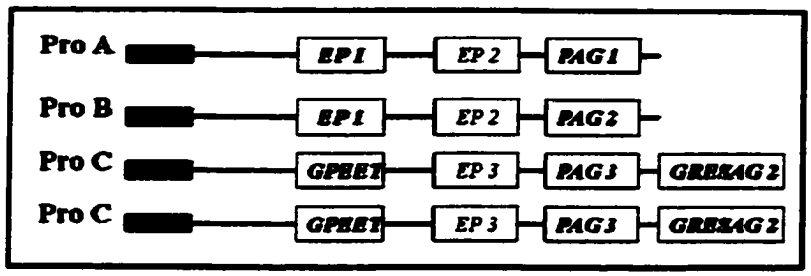


Figure 1.4. ***Map of procyclin genes.***

The expression sites are labelled Pro A, Pro B and Pro C. EP/PAG 1 and EP/PAG 2 are present as single copies, while GPEET/ PAG 3 locus exist as two copies in the diploid genome [Roditi and Clayton, 1999].

Transcription of both procyclin and *vsg* genes occurs from both their respective promoters in the BSF as well as in the procyclic forms of the parasite, clearly indicating that the stage-specific expression of these glycoproteins is regulated post-transcriptionally [Pays *et al.*, 1990]. Initiation of transcription of procyclins is 6- to 10 – fold higher in procyclic forms than in BSF [Pays *et al.*, 1990]. Unlike the *vsg* genes, transcription of procyclin genes can occur from two or more procyclin expression sites [Mowatt and Clayton, 1988]. Stage specific expression of procyclins in the insect forms of trypanosomes is regulated at various levels: at the level of mRNA stability [Hotz *et al.*, 1998] and at the post-transcriptional level [Vassella *et al.*, 2000]. In addition, expression of the procyclins is found to change with extracellular signals, as is evident from *in vitro* evidence that both glycerol and oxygen can regulate their expression [Vassella *et al.*, 2000].

The individual forms of procyclin are not expressed uniformly on the surface of the parasite. The ratio of expressed EP- and GPEET-procyclins is found to vary between

different cell lines *in vitro* [Bütikofer *et al.*, 1997]. During the life cycle of the parasite in the tsetse fly vector, the ratio of EP- and GPEET- procyclins expressed on the surface of the trypanosomes changes, depending on the stage of the infection [Vassella *et al.*, 2000; Acosta-Serrano *et al.*, 2001]. In the earlier stages of infection, i.e. at day three, a phosphorylated form of GPEET is the major procyclin expressed. By the seventh day, GPEET disappears and several isoforms of EP-procyclins, all of which are N-glycosylated, are expressed on the surface of the parasite. Thus GPEET-procyclin is repressed after 7-9 days in the fly, just as the parasites start to appear in the ectoperitrophic space. This suggests that GPEET has a specific function in the host-parasite interaction during differentiation and establishment of midgut infection [Vassella *et al.*, 2000]. Repression of GPEET-procyclin is thought to be post-transcriptionally regulated by the GPEET 3' UTR. It has also been observed that repression of GPEET expression occurs both *in vitro* and *in vivo* with similar kinetics. It is clear that though procyclins are antigenically conserved and do not demonstrate antigenic variation like VSG, they express different isoforms at different stages of the life cycle, thereby presenting a dynamic mosaic of surface molecules to the tsetse fly. The differential expression patterns exhibited by these surface glycoproteins in the insect vector implies that they play an important role in the parasite life cycle in the tsetse fly vector.

EP- procyclins have a 31- amino acid N-terminal domain consisting of a N-glycosylation site at Asn^{29}, followed by a repeat EP sequence (22-30 repeats) in its C-terminal domain and a 23 amino acid hydrophobic tail. The EP repeats comprise almost 40% of the polypeptide portion of the molecule [Roditi *et al.*, 1987; Richardson *et al.*, 1988]. There is an N-terminal signal peptide to direct the EP-protein to the ER and a C-

terminal signal for attachment of the GPI membrane anchor. The single N-glycosylation site of the EP-procyclins has a conserved $Man_5GlcNac_2$ structure [Treumann *et al.*, 1997; Mehlert *et al.*, 1998; Hwa *et al.*, 1999]. It was recently shown that though wild type procyclic forms have the capacity to synthesize high mannose structures from $Man_5GlcNac_2$ to $Man_9GlcNac_2$, they may have modified the N-glycan expression pathway to synthesize the conserved $Man_5GlcNac_2$ structure as an adaptive feature while differentiating from BSF [Hwa and Khoo, 2000]. The EP-procyclins have an polyanionic, extended rod-like structure, 14-18 nm in length and 0.9 nm in diameter which interdigitates between and extends beyond the VSG molecules in intermediate forms coexpressing both the surface coats during differentiation from BSF to insect forms [Roditi *et al.*, 1989]. Identification and characterisation of the three different isoforms of the EP-procyclins has been difficult as they have similar peptide sequences and are difficult to detect by conventional protein staining or by absorption at 280 nm. To overcome this difficulty, procyclins isolated directly from parasites taken from tsetse flies have been studied in a novel approach using mass spectrometry. Different isoforms of EP-procyclins were characterised by Acosta-Serrano *et al.*, 1999, by a proteomics approach using MALDI-TOF-mass spectrometry, after the removal of the GPI-anchor. They showed that not only different isoforms of EP-procyclins were expressed, corresponding to the three different classes of the genes encoding them, but also there was allelic variability of EP-procyclins, differing in the lengths of their EP-repeats. They were also able to detect a new non-glycosylated isoform of EP-procyclin. It can be summarised that all isoforms of EP-procyclins have similar polypeptide sequences with minor changes in their N-terminal domain. They differ in their number of EP repeats.

They are not phosphorylated. More than 90% of EP-procyclin isoforms are N-glycosylated (with the exception of EP-2 procyclin that does not have a N-glycosylation site) and the parasites have the ability to switch EP-procyclin expression to various isoforms (maybe as a response to the interactions with lectins in the tsetse midgut) [Ruepp *et al.*, 1997; Acosta-Serrano *et al.*, 1999; Acosta-Serrano *et al.*, 2000].

The GPEET- procyclins have up to six repeats of glycine – proline – glutamic acid - glutamic acid – threonine in their sequences. GPEET-procyclin exhibits different post-translational modifications when compared to that of EP-procyclins: GPEET-procyclins are not N-glycosylated and they express a phosphorylated isoform, with six out of the seven threonine residues being phosphorylated [Bütikofer *et al.*, 1997; Mehlert *et al.*, 1999]. It is interesting that both the precursors of EP- and GPEET- procyclins have highly conserved N-terminal signal peptides and hydrophobic C-terminal peptides. Apart from that, there are only two short stretches of identity between the EP- and GPEET- proteins [for alignment see Ruepp *et al.*, 1997]. A schematic representation of the two major classes of procyclins is shown in Figure 1.5.

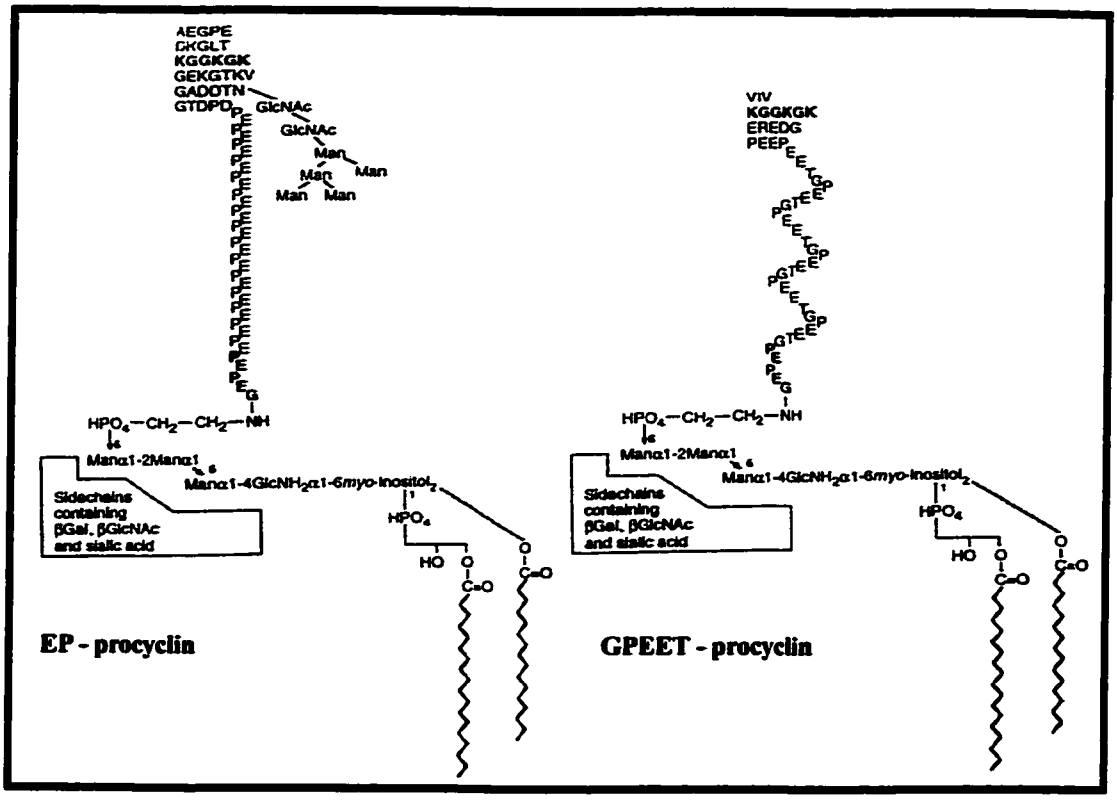


Figure 1.5. ***Schematic representation of EP-procyclin and GPEET-procyclin [Treumann et al., 1997].***

Both EP- and GPEET – procyclins are GPI anchored proteins [Bütikofer *et al.*, 1997] with similar negatively charged GPI anchors exhibiting an unusual structure of a large, complex branched side chain of carbohydrates containing on average, nine galactose, nine N-acetylglucosamine and five sialic acid residues [Ferguson *et al.*, 1993]. The complex side chain is added after the transfer of the GPI precursor to the protein and is thought to be the sialic acid acceptor for a cell surface trans-sialidase [Ferguson *et al.*, 1993]. This complex modified GPI anchor glycan structure forms a glycocalyx around the insect form parasites. The anchor structure is the largest so far described in any species of organism.

It is probable that procyclins play an important role in the life cycle of the parasite in the insect vector. Nevertheless, the precise functions associated with these surface molecules have not yet been resolved. Several hypotheses have been proposed regarding the relevance of these glycoproteins [Roditi *et al.*, 1989; Roditi and Pearson, 1990; Stebeck and Pearson, 1994; Roditi *et al.*, 1998; Acosta-Serrano *et al.*, 2001; Pearson, 2001]. Procyclins are important for establishment of infections in the vector, as it has been shown that EP-null mutants cannot establish heavy infections in the tsetse midgut [Ruepp *et al.*, 1997]. One major function hypothesised for the sialated, branched, polylactosamine side chains of the GPI-anchor of procyclins is the formation of a glycocalyx around the parasite to protect the membrane of the procyclic forms in the tsetse fly midgut [Ferguson *et al.*, 1993; Treumann *et al.*, 1997]. This is plausible as procyclins have a unique protease - resistant extended EP or GPEET structure. However, the N-terminal domains of the procyclins taken directly from parasites in tsetse flies were found to be quantitatively removed [Acosta-Serrano *et al.*, 2001] implying that tsetse proteases cleave the procyclins during parasite development in the midgut. The C - terminal domains, containing the extended repeat structures of the procyclins were nevertheless resistant to the tsetse proteases suggesting that one of their main functions is to protect the parasite surface from midgut proteases in the fly [Acosta-Serrano *et al.*, 2001]. It has also been hypothesised that the cleaved procyclin N-terminal fragments could play a role in cell signalling [Acosta-Serrano *et al.*, 2001]. Molecular interactions of these expressed glycoproteins with lectins found in the tsetse fly midgut are thought to be responsible for either parasite differentiation and survival [Maudlin and Welburn, 1988; Maudlin and Welburn, 1994] or paradoxically, cell death [Pearson *et al.*, 2000].

The N-glycans of the EP-procyclins have been shown to interact with lectins *in vitro*, thus they could serve as ligands for lectins postulated to be present in the tsetse. Such interactions could result in either cell differentiation along the route to the mammal-infective metacyclic forms or apoptosis to maintain the threshold density of these parasites in the midgut. Another major function postulated for the procyclins is in tissue tropism of the parasites in the vector. Species of the subgenus *Trypanozoon* differentiate into metacyclic forms in the salivary glands of the tsetse vector, whereas species of the subgenus *Nannomonas* differentiate in the mouthparts of the tsetse fly. Thus, differences between these two subgenus in the procyclin structures may reflect these differences tissue tropisms.

1.5. *Trypanosomes of the subgenus Nannomonas - neglected parasites*

African trypanosomes of the subgenus *Nannomonas*, pathogens of domestic animals in sub-Saharan Africa, have not been as extensively studied as *T. brucei* spp. (subgenus *Trypanozoon*). As a rule, BSF trypanosomes are isolated from original field hosts by inoculating lab rodents that relatively soon develop parasitaemia. Of the two pathogenically important species belonging to the subgenus *Nannomonas* (*T. congolense* and *T. simiae*), only isolates of *T. congolense* (which causes lethal wasting disease of cattle) can infect laboratory rodents and proliferate. *T. simiae* has been largely neglected as the BSF of these species do not infect rodents and can be grown only in pigs. PCF however, can be established from BSF by transformation *in vitro* [Pearson *et al.*, 1987] and by isolating the parasites directly from tsetse [Gray *et al.*, 1981; Dukes *et al.*, 1991]. These studies paved the way for comparative studies. Tsetse flies in both east and west

Africa can harbour *Nannomonas* infections [Mihok *et al.*, 1994] and coupled with their wide host range and the fact that their virulence depends on the species of transmitting tsetse [Janssen *et al.*, 1974; Moloo *et al.*, 1992], there emerges a complex pattern of pathogenesis. Though *T. simiae* is morphologically similar to *T. congolense*, the first biochemical evidence of them being distinct was reported by Gashumba *et al.*, in 1986. These authors showed that the isoenzyme patterns of BSF of the two species, collected at peak parasitaemia, were noticeably different. Further molecular characterisation (using DNA sequence comparisons) of these species also revealed that they are genetically distinct [Majiwa and Webster, 1987; Garside and Gibson, 1995].

Although procyclins have been extensively characterised from *T. brucei*, only one procyclin analogue has been identified from *T. congolense*; the glutamic acid – alanine rich protein (GARP) [Beecroft *et al.*, 1993; Bayne *et al.*, 1993]. As with the procyclins characterised from *T. brucei*, GARP is a stage-specific, immunodominant, acidic, surface exposed, GPI-anchored glycoprotein [Beecroft *et al.*, 1993; Bayne *et al.*, 1993]. GARP has a completely unrelated polypeptide sequence when compared to *T. brucei* procyclins. Also in contrast to procyclins from *T. brucei*, GARP has a high alanine and low proline content, lacks an N-glycosylation site and has no extensive dipeptide or pentapeptide repeats [Bayne *et al.*, 1993]. The loci encoding GARP have recently been characterised [Rangarajan *et al.*, 2000]. In the diploid genome of *T. congolense*, GARP genes are arranged in two loci, *GARP1* and *GARP2*, on the same chromosome. The *GARP1* locus contains a single GARP gene, while the *GARP2* locus exhibits allelic variation with one allele having six genes and the other ten. Similar to that of the procyclin genes in *T. brucei*, a sequence related to *ESAG4* has been identified downstream of one of the

GARP loci. In contrast to *vsg* genes or EP- and GPEET-procyclin genes in *T. brucei*, genes encoding GARP are transcribed by an α-amanitin sensitive polymerase [Graham *et al.*, 1996; Downey and Donelson, 1999]. They are however, transcribed as polycistronic units similar to the transcription of procyclin genes in *T. brucei*.

No procyclin analogues from other members of the subgenus *Nannomanas* have been characterised. However, it has been noted that there are immunological similarities between the surface molecules expressed by *T. congolense* and *T. simiae* PCF, as monoclonal antibodies directed to the surface of *T. congolense* cross-react with the surface of the *T. simiae* procyclics [Beecroft *et al.*, 1993]. Though the GARP gene is highly conserved within the subgroups of *T. congolense* [Asbeck *et al.*, 2000], it was not detected in clones of *T. simiae* or *T. godfreyi* [Garside and Gibson, 1995], suggesting that GARP may not be present throughout the subgenus *Nannomonas*.

GARP has been shown to be a functional analogue of EP-procyclins as it can compensate for them: when the GARP gene is introduced into an EP-knockout mutant of *T. brucei* procyclic forms these parasites are able to establish heavy midgut infections in the tsetse fly whereas the EP - null mutant cannot [Ruepp *et al.*, 1999]. Whether or not the analogous molecules in *T. simiae* will have the same ability is not known. Indeed, the surface molecules of *T. simiae* PCF have not been examined prior to the work reported in this thesis.

1.6. *Control of trypanosomes and trypanosomiasis*

Control of trypanosomiasis as it stands today, is dependent on chemotherapy and vector control. Due to antigenic variation exhibited by the BSF, the development of an effective vaccine has been thwarted, both practically and intellectually.

Pentamidine [for review see Sands *et al.*, 1985] and Suramin [Voogd *et al.*, 1993], developed in the 1920s remain the drugs of choice for treatment of the early phase of sleeping sickness, before the disease manifests in the central nervous system. These drugs however, are unable to cross the blood-brain barrier and thus are not suitable for late-stage sleeping sickness. The chemotherapeutic agent used for the late phase of the disease, is an arsenic-based formulation developed in 1949, Melarsoprol [Friedheim, 1949]. Unfortunately, Melarsoprol causes various adverse side effects, including encepholopathic syndrome, neuropathies and frequent relapse of the infection after treatment. Eflornithine (a polyamine synthesis inhibitor), an analogue of ornithine, is the first new effective drug developed since the 1940's [Khonde *et al.*, 1997; Smith *et al.*, 1998]. It was accidentally discovered during a quest for anti-cancer drugs. However, Eflornithine is less effective than Melarsoprol in the late stage of Gambian sleeping sickness. The other major disadvantage of Eflornithine is its cost, US$ 300 per patient. The drug can be effectively used with Gambian sleeping sickness patients who undergo a relapse following Melarsoprol therapy and has been given the name "the resurrection drug". Another new drug for Gambian sleeping sickness is Presnisolone, which reduces the risk of Melarsoprol-induced encephalopathy and mortality [Smith *et al.*, 1998]. In general, compounds that interfere with polyamine biosynthesis or function have been

valuable as therapeutic agents against African trypanosomes [Marton and Pegg, 1995; Denise and Barrett, 2001].

Limitations of the existing chemotherapeutic agents for trypanosomiasis are predominantly drug-associated toxicity and emergence of drug resistant pathogenic strains [Anene *et al.*, 2001; Geerts *et al.*, 2001]. One major breakthrough recently has been the discovery that trypanosomes have the capacity to synthesize fatty acids via an unique biosynthetic pathway which preferentially incorporates myristate (and not other lipids) into the VSG GPI-anchor. This GPI-myristoylation appears to be essential for the survival of the parasites in the bloodstream of the mammalian host, making it a potential chemotherapeutic target [Morita *et al.*, 2000]. Though there appears to be a few newly elucidated drug targets in BSF [for reviews see Barrett *et al.*, 1999; Keiser *et al.*, 2001] that have the potential to be exploited, research on and development of new chemotherapeutic agents has been hindered due to economic reasons. Pharmaceutical companies are reluctant to invest in the development of new chemotherapeutic agents as costs and profits associated with new formulations, even those that have shown promise *in vitro*, seem to be prohibitive. It is appalling that currently no new trypanocidal drug are in Phase I – III of clinical trials [Denise and Barrett, 2001; Keiser *et al.*, 2001].

A widely adapted approach to trypanosomiasis control has focused on tsetse flies [for review see Schofield and Maudlin, 2001; Allsopp, 2001]. Before the discovery of organochlorine insecticides, the strategies employed were extensive clearing of fly-infested vegetation and shooting of wild game animals on which flies fed and which acted as a reservoir of the parasites. The advent of DDT and other persistent insecticides resulted by the mid-1950s in programs which included ground and aerial spraying as a

relatively inexpensive means of tsetse control [for review see Grant, 2001]. In the last decade, environmentally favourable techniques such as development of visual or odour-baited traps and targets that are impregnated with insecticides have gained momentum. Recently, since the development of pyrethroids, insecticides of low toxicity to mammals, application of these insecticides to the animals on which flies feed opened up the path for cattle "dip" and "pour-on" technique. These methods protect the cattle not only from tsetse but also from ticks and other biting flies [Allsopp, 2001; Grant, 2001]. An ambitious approach that does not involve insecticides and that has been used successfully in reducing *Glossina* spp., is the sterile insect technique, which employs sterilised male tsetse to reduce the incidence of naturally fertilized female flies [Aksoy *et al.*, 2001; Allsopp, 2001; Schofield and Maudlin, 2001]. Finally, manipulation of the midgut endosymbionts of the tsetse fly has been suggested as a futuristic means to interrupt parasite transmission [Aksoy *et al.*, 2001].

In spite of the various tactics both suggested and used over the years, trypanosomiasis still remains one of the major causes of human illness and economic destruction due to infection of livestock, in sub-Saharan Africa.

1.7. *Purpose of this thesis*

One strategy for control of trypanosomiasis that could be exploited is the concept of a transmission blocking vaccine. Here, an intimate knowledge of parasite-vector interactions would be essential for developing novel interference strategies. The biochemistry of interactions between tsetse fly molecules and insect forms of the parasites has to be sufficiently understood in order devise detailed interventions in the

parasite life cycle. Thus, the focus of the work presented in this thesis was on the procyclic (midgut) forms of *T. simiae* with a focus on the major surface antigens.

PCF were used for *in vitro* experiments. Procyclic forms grown *in vitro* and *in vivo* appear to be essentially identical as they have similar ultrastructural characteristics and they appear to utilise the same respiratory substrates [Vickerman *et al.*, 1988]. Moreover, high- resolution, two-dimensional polyacrylamide gel electrophoresis analysis revealed that the proteins expressed by the PCF and the procyclic forms from the tsetse midgut were qualitatively similar [Pearson *et al.*, 1987].

The surface molecules, which form the protective coat expressed by the members of the subgenus *Nannomanas* were studied in this project. The surface molecules of two closely related species of this subgenus, *T. simiae* and *T. congolense* were compared in order to better understand their biochemical and immunological features that may have relevance in parasite-vector interactions.

2. Materials and methods

2.1. *Trypanosomes*

BSF of *T. b. brucei* 427.1 [Cross and Manning, 1973] and *T. congolense* K45/1 (derived from *T. congolense* STIB744) were obtained from Dr. R. Brun (Swiss Tropical Institute, Basel, Switzerland). BSF of *T. simiae* CP11 [Zweygarth and Rötcher, 1987] were obtained from E. Zweygarth, Veterinary Laboratories, Kabete, Kenya. Procyclic culture forms (PCF) of the above trypanosomes were derived from their corresponding BSF by transformation at 27 °C as previously described [Brun and Schönberger, 1979]. All PCF were maintained at 27 °C in a modified minimum essential medium (MEM) containing Earle's salts/ 25 mM HEPES/ 10-20% foetal bovine serum (FBS)/ 1% non-essential amino acids/ 2 mM glutamine/ 2.6 μg ml^{-1} hemin/ 60 μM proline and 200 μm hypoxanthine [PCF medium; Fish *et al.*, 1989]. In addition, 50 units ml^{-1} of penicillin – streptomycin were added to the PCF medium. However the antibiotics were not included in PCF medium used for the growth and maintenance of *T. simiae*.

2.2. *Biosynthetic labeling with ^{35}[S]-methionine*

Biosynthetic labeling of methionine-containing proteins was performed as previously described [Pearson *et al.*, 1987] with several minor modifications. Essentially, *T. simiae* and *T. congolense* PCF were adapted to Dulbecco's Modified Eagle's Medium containing glucose and pyridoxine hydrochloride (GIBCO BRL®, Burlington, ON, Canada), supplemented with 2 mM glutamine/ 200 μM hypoxanthine/ 2.6 μg ml^{-1} hemin and 20% dialyzed FBS. During their exponential growth phase the

parasites were harvested by centrifugation at 850 x g for 10 min at RT and washed once with the above medium without FBS. Washed PCF (2×10^7) were resuspended in 0.5 ml of the above medium without methionine and the suspension was added to a 50 ml tissue culture flask containing 4.5 ml of methionine-free medium containing 20% dialyzed FBS. The flask was gassed with 5% CO_2 in air and then 100μCi ^{35}[S]-methionine (Amersham Pharmacia Biotech Inc., Baie de Urfe, Quebec, specific activity >800 Ci $mmol^{-1}$) were added to the medium. The flasks were incubated at 27 °C for 18 hr and PCF harvested by centrifugation at 850 x g for 10 min at 20 °C. For 2-dimensional gel electrophoresis, the labelled PCF were lysed in SDS-mix [Pearson *et al.*, 1987] and the solubilized lysates stored at -20 °C until further use.

2.3. *High resolution 2-D gel electrophoresis*

Multiple 2-D gel analysis using the ISO-DALT® system [Anderson and Anderson, 1979] was performed as described by Pearson *et al.*, [1987]. For each parasite lysate 2 x 10^6 cpm were loaded onto the pre-focused first dimension tube gels containing pH range 3-10 ampholines (Pharmalyte 3-10, Amersham Pharmacia, Upsala, Sweden). First dimension isoelectric focusing was conducted at 800 V for 16 hours. Following first dimension electrophoresis, the tube gels were equilibrated for 15 min at room temperature in equilibration buffer; 10% (v/v) glycerol/ 1.5% (w/v) Tris-base/ 2% (w/v) SDS/ 0.075% (w/v) DTE/ 0.1% (w/v) Bromophenol blue and were mounted onto 5% - 15% gradient SDS-PAGE slab gels. Second dimension electrophoresis was performed at 4°C at 1 Amp until the dye front was about 1 cm from the bottom of the gel (for about 5 hours). The gels were dried and exposed to Kodak Biomax™ MR film (Eastman Kodak

Company, Rochester, NY, USA) with intensifying screens for 6 days at –80 °C. Scanned autoradiographs were converted to JPEG files using Photoshop™ 5.5 graphics software (Adobe Systems Inc., San Jose, CA). The autoradiograph profiles were analysed using 2-D gel image analysis software, Melanie 3 (GeneBio, Geneva Bioinformatics SA, Geneva, Switzerland).

2.4. *Southern blot analysis*

To determine whether the gene encoding GARP of *T. congolense* was present in *T. simiae* CP11, low – moderate stringency, in-gel Southern blot analysis was performed using a PCR fragment corresponding to the *T. congolense* GARP coding region [Bayne *et al.*, 1993] as the probe. The probe was obtained in the plasmid pGARP-PCR [Hehl *et al.*, 1995] and was a gift from Dr. Isabel Roditi, Bern, Switzerland. The GARP probe was amplified from the plasmid by PCR using standard universal sequencing primers. Genomic DNA from PCF of *T. simiae* CP11, *T. congolense* K45/1 and *T. b. brucei* 427 was isolated from lysates of the parasites using standard methods [Sambrook *et al.*, 1989]. DNA (10 µg from each parasite) was digested in an Eppendorf microcentrifuge tube overnight at 37 °C with 100 units of the restriction enzyme Bam HI (New England Biolabs, Beverly, MA, USA) followed by a further 4 hour digestion with another 100 units of enzyme at 37 °C. The digested DNA was precipitated by addition of 2.8 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate pH 5.2, followed by centrifugation at 14,000 x g for 1 hour at 4 °C. The precipitate was resuspended in 30 µl of distilled water and the digest was electrophoresed for 18 hours at 10 volts on a 0.6% agarose gel. Lambda DNA-BstE II-digest (New England Biolabs) was used as size

standards. The agarose gel was treated with denaturation buffer (1.5 M NaCl/ 0.5 M NaOH for 30 min X 2) followed by neutralisation with 1.5 M NaCl/ 0.5 M Tris-HCl pH 7.5 for 15 min X 2 [Sambrook *et al.*, 1989] and dried using a BioRad model 483 slab gel dryer (BioRad Laboratories, Hercules, CA, USA) for 30 min without heat followed by 90 min at 80 °C. Prior to probing, the gel was rehydrated and treated with hybridization buffer (0.25 M Na_2HPO_4, pH 7.2/ 7% (w/v) SDS) for 5 min at 55 °C [Bridge *et al.*, 1998]. The gel was probed overnight at 55 °C with 25 ng of a PCR fragment corresponding to the *T. congolense* GARP coding region [Bayne *et al.*, 1993] labelled with [α^{32}-P] dATP (specific activity 3000 Ci/mM). The gel was further washed with 0.25 M Na_2HPO_4, pH 7.2/ 7% SDS (2 X 30 min) at 55 °C. Radioactivity in the gel was subsequently measured using a STORM 820 storage phosphor imaging system (Molecular Dynamics, Sunnyvale, CA, USA).

2.5. *Monoclonal antibodies and antisera*

The mAb TC 491 (IgG_3) used in this study is specific for procyclin-like molecules on the surface of *T. congolense* [Beecroft *et al.*, 1993]. This mAb binds most strongly to a *T. congolense* protease-resistant surface molecule [Bütiköfer *et al.*, in press] but also binds to epitopes on GARP [Beecroft *et al.*, 1993] and likely recognizes a carbohydrate epitope. MAb TS 126 (IgM) was raised against living *T. simiae* PCF using immunisation and screening procedures as previously described for *T. congolense* [Beecroft *et al.*, 1993] with minor modifications in that the ClonaCell-HY™ system (StemCell Technologies Inc., Vancouver, B.C.) was used for hybridoma derivation and growth. Essentially, female BALB/c mice were immunized by intraperitoneal injections of

1 X 10^7 live *T. simiae* CP11 PCF, followed by a booster injection after fifteen days. Three days later the mouse spleen cells were fused with X63-Ag8.6.5.3 BALB/c parental myeloma cells as described by Pearson *et al.*, [1980]. The cell fusion mixture was diluted and plated in a semi-solid methylcellulose matrix containing HAT selective medium and B-cell growth factors, allowing single-step selection and cloning of hybridomas. The tissue culture supernatants from the growing hybridomas were screened by ELISA using the immunizing antigen to identify positive hybridomas and then human transferrin to eliminate non-specific "sticky" mAbs (described below). Similarly, several mAbs were generated against reverse-phase HPLC-purified molecules from *T. simiae* CP11 after deglycosylation with anhydrous trifluoromethane sulfonic acid (TFMSA; see below). A mixture of mAbs L98 and L157 was used to monitor the reverse-phase HPLC fractions for kinetoplastid membrane protein-11 [KMP-11; Tolson *et al.*, 1998] that is found in all kinetoplastid parasites [Stebeck *et al.*, 1996] and which elutes from reverse-phase HPLC column just prior to the procyclins of *T. brucei*.

Various polyclonal antisera were also used in this study. A rabbit antiserum was derived against *T. congolense* GARP that had been deglycosylated using anhydrous TFMSA (procedure for deglycosylation explained below). A rabbit antiserum specific for proaerolysin toxin of Aeromonas *hydrophila* was produced as previously described [Howard and Buckley, 1985] and was a gift from Dr. J.T. Buckley (Dept. of Biochemistry, University of Victoria, B.C., Canada). An antiserum was also prepared against a synthetic peptide, RREHYTVGR, predicted to be a B-cell epitope (see chapter 4). This peptide was coupled to a carrier protein, KLH (see below). The KLH-synthetic

peptide was used as the immunizing antigen, at a concentration of 25 µg per injection (X 3 injections), to raise mouse antisera directed against the peptide.

2.6. *Purification of the surface molecules of T. simiae and T. congolense PCF*

Molecules were purified from *T. simiae* and *T. congolense* PCF by reverse-phase HPLC using a semi-preparative octyl-Sepharose column (1 cm x 25 cm) and a procedure modified from one originally developed for purification of procyclins from *T. brucei* spp. [Ferguson *et al.*, 1993]. PCF were harvested during their exponential growth phase by centrifugation at 850 x g for 10 min at RT and washed once with PCF medium without FBS. The cell pellets containing 2 x 10^{10} cells were delipidated by two extractions with 8 ml of chloroform/methanol/water (1:2:0.8, by vol) by centrifugation at 4000 x g for 10 min at RT. The insoluble delipidated residue was extracted twice with 5 ml of 9% butan-1-ol (v/v) in distilled water, with sonication, on ice, followed by centrifugation at 4000 x g for 10 min at RT. The pooled supernatants were lyophilized. The dried, 9% butan-1-ol extracts were dissolved in 5 ml of 0.1 M ammonium acetate/ 5% propan-1-ol (v/v), vortexed, centrifuged at 4000 x g for 10 min at RT and the supernatants loaded onto the octyl-Sepharose column that was pre-equilibrated with 0.1 M ammonium acetate/ 15% propan-1-ol (v/v). Reverse-phase HPLC was performed at a flow rate of 0.3 ml/min using a linear gradient from 15% propan-1-ol in 0.1 M ammonium acetate to a final composition of 70% propan-1-ol (v/v) in 0.1 M ammonium acetate over 860 min. Fractions (2.9 ml) were collected and screened by indirect ELISA (see below) with surface directed mAbs : anti-*T. simiae* CP11 mAb TS 126, anti-*T. congolense* K45/1 mAb TC 491 and anti-KMP 11 mAb L98/L157 mixture as primary antibodies. The

immunoreactive fractions were pooled, concentrated by vacuum evaporation (EYELA vapor mix S-10, Tokyo Rikakikai Company Ltd., Tokyo, Japan), lyophilized and stored frozen at –20 °C until further use. The lyophilized preparation was dissolved in 500 µl of 9% propan-1-ol in water (v/v) containing 0.1% Triton® X100 for SDS-PAGE analysis and immunoblotting experiments.

2.7. *Enzyme-linked immunosorbent assay* (ELISA)

Indirect ELISA using solid-phase adsorbed antigens was performed essentially as previously described [Richardson *et al.*, 1986]. HPLC fractions (60 µl of each of the 2.9 ml fractions) were coated onto wells of ELISA plates by drying overnight at 37 °C. Ascites fluids containing mAb TS 126, mAb TC 491 or mAb L98/L157 mixture were used as primary antibodies at a dilution of 1:1000. A 1:2,500 dilution of alkaline-phosphatase-conjugated goat anti-mouse IgG/IgM (Caltag, South San Francisco, CA, USA) was used as the secondary antibody. The substrate used was p-nitrophenyl phosphate (Sigma Chemical Company, Mississauga, ON, Canada) and absorbance at 405 nm was read after 20 minutes using an automated EIA plate reader (Model EL 310, Bio-Tek Instruments, Inc., Burlington, VT, USA).

Isotyping of the mAbs was performed using the tissue culture supernatants of the hybridomas in an antigen capture ELISA technique. A polyspecific goat anti-mouse immunoglobulin antiserum was used as the capture antibody (Caltag, South San Francisco, CA, USA) and alkaline phosphatase conjugated-goat anti-mouse IgG1/ IgG2a/ IgG2b/ IgG3/ IgA/ IgM (Caltag, South San Francisco, CA, USA) antisera were the isotyping reagents. They were used at a dilution of 1:2000 to 1:3000. The substrate used

was p-nitrophenyl phosphate (Sigma Chemical Company, Mississauga, ON, Canada) and absorbance at 405 nm was read using an automated EIA plate reader (Model EL 310, Bio-Tek Instruments, Inc., Burlington, VT, USA).

2.8. *Immunofluorescence*

Indirect immunofluorescence was performed on suspensions of live PCF and on fixed-permeabilized parasites [Pearson *et al.*, 1981]. The parasites were fixed using either acetone or 4% (w/v) formaldehyde. Tissue culture supernatants containing mAb TS 126 and mAb TC 491 were used at a 1:4 dilution as primary antibodies. The mouse antiserum directed against the KLH-coupled *T. simiae*-specific synthetic peptide (described above) was used at a 1:100 dilution as the primary antibody. A 1:40 dilution of FITC-labelled goat anti-mouse IgG/IgM (Caltag, South San Francisco, CA, USA) was used as secondary antibody for detection of both mAbs and polyclonal antibodies. For fixed parasites, SlowFade™ (Molecular probes Inc., Eugene, OR, USA) was added to prevent fading of fluorescence emission. Immunofluorescence was observed using a Zeiss Standard microscope fitted with an epifluorescence attachment and an oil-immersion 100 X Neofluor objective.

2.9. *One-dimensional polyacrylamide gel electrophoresis*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [Laemmli, 1970] using a Mini-Protean II minigel apparatus (BioRad Laboratories, Hercules, CA, USA). Unlabelled, biotin-labelled or radiolabelled HPLC-purified molecules from *T. congolense* and *T. simiae* PCF were

separated at 100 volts using a 0.75 mm, 3% stacking gel and a 10% resolving gel. Rainbow™ high molecular weight colored markers (14.3 - 220 kDa, Amersham Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada) were run on each gel. The gels were subsequently stained with GelCode® Blue according to the manufacturer's instructions (Pierce Chemical Company, Rockford, IL, USA), with silver stain [Merril *et al.*, 1984], or with Stains-All™ (Sigma Chemical Company, Mississauga, ON, Canada) or were used for immunoblotting, autoradiography or fluorography. Stains-All™ was used according to Green *et al.*, [1973] with minor modifications. Essentially, the gels were gently agitated in 25% (v/v) isopropanol at 50 °C for 15 min, 1% (v/v) Triton® X100 at 50 °C for 30 min, then rinsed with distilled water. They were stained overnight in the dark at RT by agitating gently in 0.02% (w/v) Stains-All™ in 5% (w/v) formamide/ 25% (v/v) isopropanol and 1.5 M Tris-HCl pH 8.8. The gels were finally destained with distilled water.

Digital images of stained gels were captured by scanning at 300 dpi using a colour scanner (UMAX Astra 3400, Fremont, CA) after briefly rinsing the gels in distilled water. The images were manipulated and stored as JPEG and/or TIFF files using Photoshop™ 5.5 graphic software (Adobe Systems Inc., San Jose, CA).

2.10. *Immunoblotting*

Proteins separated by SDS-PAGE were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P™, Millipore, Corp., Bedford, MA, USA) for 30 min at 90 volts. Antigens were subsequently detected by the relevant mAb or antiserum, followed by horseradish peroxidase-labelled anti-murine or

anti-rabbit IgG/IgM specific secondary antibodies (Caltag, South San Francisco, CA, USA). Optimal mAb dilutions (1/2 for hybridoma tissue culture supernatants and 1/10,000 for ascites or antiserum) and secondary antibody dilution (1/50,000) were determined by titration. SuperSignal® Dura enhanced chemiluminescence substrate (Pierce Chemical Company, Rockford, IL, USA) was used according to the manufacturer's instructions. The developed blots were exposed to Kodak Biomax™ MR film (Eastman Kodak Company, Rochester, NY, USA).

2.11. *In-situ oxidation and labeling of glycoproteins with biotin hydrazide*

SDS-PAGE-separated proteins were blotted onto PVDF membranes. Blots were washed extensively with PBS prior to oxidation of the oligosaccharide moieties of the glycoproteins with 10 mM sodium meta-periodate in 0.1 M sodium acetate buffer pH 5.5, for 30 min at 0 °C in the dark. Blots were incubated with 15 mM glycerol for 5 min at 0 °C to quench the oxidation reaction and then washed with PBS and incubated with 5 mM biotin hydrazide (EZ-Link™, Pierce Chemical Company, Rockford, IL, USA) for two hours at RT with agitation in the dark [O'Shannessy, 1987]. Blots were then washed extensively with PBS/ 0.1% (v/v) Tween®-20 for 5 min X 3, followed by a wash for 15 min and blocked with 5% (w/v) skim milk powder (DIFCO, Becton Dickinson, Sparks, MD, USA) in PBS. Biotinylated molecules were detected using a 1:5000 dilution of streptavidin labeled with horseradish peroxidase (Cedarlane Laboratories Ltd., Hornby, ON, Canada). Blots were developed with SuperSignal® Dura enhanced chemiluminescence substrate (Pierce Chemical Company, Rockford, IL, USA) according

to the manufacturer's instructions. The developed blots were exposed to Kodak Biomax™ MR film (Eastman Kodak Company, Rochester, NY, USA).

2.12. *Biosynthetic labeling of lipid anchors with [1-^{3}H] ethan-1-ol-2-amine hydrochloride*

PCF of *T. simiae* CP11 and *T. congolense* K45/1 (5 x 10^7 in 5 ml PCF medium with 20% FBS) were added to 5 ml of fresh PCF medium, followed by addition of 50 µCi of [1-^{3}H] ethan-1-ol-2-amine hydrochloride (specific activity 290 mCi/mg; Amersham Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada) dissolved in 0.5 ml of PCF medium containing 5 mg/ml defatted BSA [Bütikofer *et al.*, 1997]. The cells were incubated at 27 °C for 18 hr, pelleted at 2000 x g for 10 min at 4 °C and washed once with ice-cold 10 mM Tris/ HCl containing 140 mM NaCl, pH 7.4 by centrifugation at 2000 x g for 15 min at 4 °C. The pellet was delipidated with chloroform/ methanol/ water (10:10:3, by vol.) by centrifugation at 2000 x g for 15 min at 4 °C and the insoluble pellet was extracted with 1 ml of 9% (v/v) butan-1-ol in distilled water. The butanol phases were combined and 20 µl were added to 10 ml of scintillation fluid (ScintiVerse® Fisher Scientific Ltd., Nepean, ON, Canada) and counted for radioactivity. The butanol extract was then lyophilized and the dried material was dissolved in 50 µl of 9% (v/v) propan-1-ol in distilled water. Radiolabeled proteins were separated by SDS-PAGE (10% resolving gels), the gels were soaked in Amplify™ (Amersham Pharmacia Biotech, Inc.), dried using a BioRad model 483 slab gel dryer (BioRad Laboratories, Hercules, CA, USA) and exposed to Kodak Biomax™ MS film (Eastman Kodak Company, Rochester, NY, USA) with intensifying screens for 7 days at –70 °C.

2.13. *Detection of proaerolysin-binding proteins*

Proaerolysin, the inactive precursor of the channel-forming protein toxin aerolysin, binds specifically and with high affinity to several GPI anchors of cell surface proteins [Nelson *et al.*, 1997]. HPLC-purified molecules from *T. simiae* and *T. congolense* were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were blocked using 5% (w/v) skim milk powder in PBS/ 0.1% (v/v) Tween® 20 for 1 hour at RT and were incubated with 50 μg of proaerolysin in 25 ml of blocking buffer for 1 hour at RT. A 1:10,000 dilution of rabbit anti-aerolysin polyclonal antiserum in the blocking buffer was used as the primary antibody. Proaerolysin and antiserum specific for proaerolysin were obtained from Dr. J. T. Buckley, Dept. of Biochemistry, University of Victoria, B.C., Canada. Anti-rabbit IgG/ IgM labeled with horseradish peroxidase (Caltag, South San Francisco, CA, USA) was used as the secondary antibody at a 1:50,000 dilution. Blots were developed with SuperSignal® Dura enhanced chemiluminescence substrate (Pierce Chemical Company, Rockford, IL, USA) according to the manufacturer's instructions. The developed blots were exposed to Kodak Biomax™ MR film (Eastman Kodak Company, Rochester, NY, USA).

2.14. *Amino acid microanalysis*

Amino acid microanalysis was performed on the pooled HPLC peaks from *T. congolense* K45/1 and *T. simiae* CP11 PCF. Amino acid microanalysis was also performed on SDS-PAGE separated, HPLC-purified molecules from *T. simiae* PCF after blotting to PVDF membrane. In this case, bands were cut from unstained membrane after

immunodetection of the bands in adjacent lanes using surface directed mAbs as mentioned before. These PVDF bands were air dried and hydrolyzed in 6 N HCl at 165 °C for 45 min under argon gas. Amino acid microanalysis was performed using an Applied Biosystems model 420 derivatizer-analyzer by Sandy Keilland at the University of Victoria Tripartite Microanalytical Center.

2.15. *Protein microsequencing*

PVDF membrane bands containing SDS-PAGE separated, HPLC-purified molecules from *T. simiae* PCF were placed directly in a pulse liquid-phase sequencer (model 470A, Applied Biosystems, Foster City, CA) and Edman sequence analysis was performed by Sandy Keilland at the University of Victoria Tripartite Microanalytical Center.

2.16. *Gel staining for mass spectrometry*

Gels were agitated gently in fixative containing 50% (v/v) ethanol/ 3% (v/v) ortho phosphoric acid for 1-4 days at room temperature, washed three x 30 min in distilled water and allowed to equilibrate in Neuhoff's solution, 16% (w/v) ammonium sulphate/ 25% (v/v) methanol/ 5% (v/v) ortho phosphoric acid [Neuhoff *et al.*, 1988] for one hour with gentle agitation. One gram of Coomassie Brilliant Blue G-250 (EM Science, Gibbstown, NJ) was sprinkled into the Neuhoff's solution and staining continued for 3-5 days. Once well-stained protein bands were visible, protein bands were cut or the intact gels were transferred into a 20% (w/v) ammonium sulphate solution for storage at 4°C . [Neuhoff *et al.*, 1988].

2.17. *Reduction, alkylation and tryptic digestion of gel-separated proteins*

Protein bands of interest from the HPLC-purified molecules of *T. simiae* and *T. congolense* PCF, separated by SDS-PAGE and stained with colloidal Coomassie Brilliant Blue, were cut from the gels and transferred to a 1.5 ml Eppendorf microcentrifuge tubes (previously autoclaved and rinsed with 50% methanol to remove any contaminants) for digestion. Protein bands were de-stained with 50% (v/v) methanol/ 5% (v/v) acetic acid, further reduced with 10 mM DTT for 20 min at 37 °C, cooled to RT and alkylated with 100 mM iodoacetamide with incubation for 20 min at RT in dark [Kinter and Sherman, 2000]. The gel pieces were washed with 0.2 M NH_4CO_3 in 50 % (v/v) acetonitrile for 15 min at RT, followed by drying the gel pieces at 37 °C for 15 min. The gel pieces were partially rehydrated with 0.2 M NH_4CO_3 and the carboxyamidomethylated protein spots were digested overnight at 37 °C with 20ng/µl modified porcine sequence grade trypsin according to the manufacturer's directions (Promega, Madison, WI). Peptides were extracted from the gel pieces using a series of elutions with 50% (v/v) acetonitrile and 5% (v/v) formic acid. The resulting pooled eluates were each reduced to a final volume of 20 µl in a Speed Vac Concentrator (Savant, Hicksville, NY) and processed for mass spectrometry.

2.18. *Peptide mass mapping by mass spectrometry*

Peptide masses were determined using matrix-assisted laser desorption ionization time-of flight (MALDI-TOF) mass spectrometry. Tryptic digest eluates were mixed with analyte solution (α-cyano-4-hydroxycinnamic acid; Aldrich, Milwaukee, WI) and spotted

onto a Voyager 100 position, stainless steel MALDI plate (Applied Biosystems, Foster City, CA). The target was inserted into an Applied Biosystems Voyager DE-STR and mass spectrometry data was acquired in delayed extraction, reflectron mode. The masses of the observed peptides were submitted to MS-Fit (Protein Prospector software package; http://prospector.ucsf.edu/) and Mascot (Matrix Science; http://www.matrixscience.com/) to perform the peptide mass fingerprint searches.

2.19. *Peptide sequencing by tandem mass spectrometry*

Samples were prepared for electrospray ionization (ESI) by desalting of peptides using glass capillary needles (Protana Inc., Staermosegaardsvej, Denmark) packed with C18 resin. Peptides were eluted from sample needles using 1.0 µL 50% (v/v) methanol/ 1% (v/v) formic acid in distilled water. Nanospray ESI was used to introduce ions into the PE-SCIEX Q-STR*i* quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). Data were managed with Bioanalyst Software version 8.7 (PE-SCIEX, Boston, MA). Peptide fragmentation data searching was performed using the Mascot MS/MS Ions Search algorithm (Matrix Science; London, UK: http://www.matrixscience.com/).

2.20. *Chemical deglycosylation with anhydrous trifluoromethane sulfonic acid*

Molecules purified from *T. simiae* PCF by reverse-phase HPLC were dissolved in 600 µl of anhydrous trifluoromethane sulfonic acid (TFMSA), (Sigma Chemical Company, Mississauga, ON, Canada) in a Reactivial™ (Pierce Chemical Company, Rockford, IL, USA). The deglycosylation reaction was performed for 2 hours at 0 °C,

with magnetic stirring in an ice bath [Edge *et al.*, 1981] and the reaction was terminated by adjusting the pH to 7.0 – 8.0 with 8 ml of 2 M Tris-base. The mixture was concentrated to a final volume of 1 ml with a Centriprep-3 concentrator, that had a molecular mass cut-off of 3,000 Da (Amicon®, Inc., Beverly, MA. USA) and was desalted by gel permeation chromatography on a Sephadex G-10 column of 10 ml bed volume. The column was eluted with distilled water and 1 ml fractions were collected and monitored for the presence of peptide bond at 230 nm. Conductivity of the fractions was also monitored using a conductivity meter (model CDM2e, Radiometer A/S, Copenhagen, NV, Denmark). The peptide containing fractions were pooled, lyophilized and stored at –20 °C until further use.

2.21. *Enzymatic deglycosylation using PNGase F1*

HPLC-purified molecules from *T. simiae* PCF were deglycosylated using PNGase F1 (Sigma Chemical Company, Mississauga, ON, Canada) according to the manufacturer's instructions, in 100 mM HEPES pH 8.6/ 1% NP40/ 1000 units of PNGase F1 overnight at 37 °C. The treated protein was precipitated with 10 X volume of ice cold acetone at – 20 °C overnight. The sample was further centrifuged at 14,000 x g for 10 min at 4 °C, the resulting pellet was evaporated to dryness in a Speed Vac Concentrator (Savant, Hicksville, NY) and taken up in 100 µl SDS-PAGE Laemmli buffer [Laemmli, 1970] for immunoblotting experiments.

2.22. *Cyanogen bromide digestion*

Molecules purified from *T. simiae* PCF by reverse-phase HPLC were dissolved in 70% (v/v) formic acid containing 10 mg cyanogen bromide (CNBr), (Eastman Kodak, Rochester, NY, USA). The solution was sparged with argon, incubated overnight at RT in the dark [Matsudaira, 1989] and the reaction terminated by diluting the digest mixture with 10 ml of distilled water. Finally, the mixture was lyophilized. Separate sets of CNBr digest experiments were performed both before and after chemical deglycosylation of the purified molecules. The resulting CNBr - cleaved peptides were dissolved in 200 µl of 0.1% trifluoroacetic acid (TFA) for peptide separation by microbore reverse-phase HPLC (see below) or in 50 µl of sample buffer for peptide separation employing peptide gels (explained in the following section).

2.23. *Peptide separation by reverse-phase microbore HPLC*

After CNBr cleavage, peptides were dissolved in 200 µl of 0.1% TFA and loaded onto a microbore, reverse-phase C_{18}, Ultrasphere ODS (5 µm), 2.0 mm X 25 cm HPLC column (Beckman Instruments Inc., Fullerton, CA, USA), that was pre-equilibrated with 0.1% (v/v) TFA/ 0.08 % (v/v) acetonitrile. The peptides were chromatographed using a linear gradient from 0.1% (v/v) TFA/ 0.08 % (v/v) acetonitrile to a final eluant composition of 100% acetonitrile over 75 min, at a flow rate of 0.2 ml/min. Fractions were monitored at 230 nm and peaks containing peptides were processed for microsequencing and amino acid microanalysis.

2.24. *Peptide separation by tricine-SDS-polyacrylamide gel electrophoresis*

Peptides were separated by polyacrylamide gel electrophoresis using a Mini-Protean II minigel apparatus (BioRad Laboratories, Hercules, CA, USA). After CNBr digestion, peptides were dissolved in 50 µl sample buffer containing 50 mM Tris-base/ 12% (v/v) glycerol/ 4% (w/v) SDS/ 2% (v/v) mercaptoethanol/ 0.01% (w/v) Coomassie Brilliant Blue G250. Electrophoresis was performed using a discontinuous Tris-tricine buffer system and a 3.9% stacking gel (0.75 mm) for 30 min at 30 volts followed by separation on a 15% resolving gel (0.75 mm) at 100 volts [Schagger and von Jagow, 1987]. Low molecular mass markers (3.4 - 66 kDa, Amersham Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada) were run on each gel. The gels were subsequently stained with GelCode® Blue according to the manufacturer's instructions (Pierce Chemical Company, Rockford, IL, USA).

2.25. *Prediction of B-cell epitope*

Protein sequence was analysed using Protean™, protein analysis software (DNAStar Inc., Madison, WI, USA). Different algorithms were used to predict characteristics such as surface probability (Emini method), hydrophilicity (Kyte-Doolittle method) and antigenic determinants (Jameson-Wolf and Hopp-Woods method). The composite graph was analysed to predict peptide sequences that were potentially strong B-cell epitopes.

2.26. *Coupling of synthetic peptide to carrier protein*

The peptide, RREHYTVGR, predicted to be a B-cell epitope, was synthesized by Daryl Hardie at the University of Victoria Tripartite Microanalytical Center. The peptide was coupled to preactivated KLH (Imject® KLH; Pierce Chemical Company, Rockford, IL, USA). Five mg of KLH (1 nmol) and a 40-fold excess of the peptide (40 nmol) were dissolved in PBS pH 7.4. The peptide was coupled to KLH using a final concentration of 1% glutaraldehyde at 4 °C for 1 hour with magnetic stirring [Harlow and Lane, 1988]. The reaction was terminated by addition of 20 mg of $NaBH_4$. The conjugate was dialysed against PBS pH 7.4 (X 3), diluted with PBS to a final concentration of 0.5 mg/ ml and stored at – 20 °C until further use. Peptide was coupled to BSA in the same fashion. This latter conjugate was used in ELISA to detect peptide-specific antibodies.

2.27. *Electron microscopy*

PCF (2×10^7) were harvested during their exponential growth phase by centrifugation at 850 x g for 10 min at RT and washed once with 0. 1M phosphate buffer pH 7.4. The washed cell pellet was resuspended in 500 µl of the primary fixative of 2.5% (v/v) glutaraldehyde/ 0.3 M NaCl in 0.1 M phosphate buffer pH 7.4. The parasites were fixed at RT for 1 hour, washed with 0.6 M NaCl in 0.1 M phosphate buffer pH 7.4 and postfixed for 1 hour at 4 °C with 1% (w/v) osmium tetroxide/ 0.3 M NaCl in 0.1 M phosphate buffer pH 7.4 [Burleigh *et al.*, 1993]. The PCF were stained *en bloc* overnight with 1% (w/v) uranyl acetate at RT, dehydrated in graded acetone, 30% – 100 % (v/v), followed by 100% propylene oxide treatment and embedding in Epon/Araldite resin with overnight polymerization at 60 °C. Ultrathin sections of the embedded samples were cut

and collected onto nickel grids. For immunogold localisation by electron microscopy, the primary fixative used was 0.26% (v/v) glutaraldehyde/ 4% formaldehyde (w/v) in 0.1 M phosphate buffer pH 7.4, followed by post fixation with 1% (w/v) osmium tetroxide, *en bloc* staining with 1% (w/v) uranyl acetate, graded acetone dehydration and propylene oxide treatment as described above. Ultrathin sections of the samples embedded in Epon/Araldite resin were cut and collected onto formvar coated nickel grids. Finally, a mouse antiserum raised against the above mentioned KLH-coupled synthetic peptide was used to probe the sections. Bound immunoglobulins were detected using anti-mouse IgG (whole molecule) coupled to 10 nm gold particles (Sigma Chemical Company, Mississauga, ON, Canada).

2.28. *First-strand cDNA synthesis*

Total RNA from lysates of *T. simiae* PCF was isolated using a mono-phasic solution of phenol and guanidine isothiocyanate: TRIzol™ reagent (GIBCO BRL®, Burlington, ON, Canada) according to the manufacturer's instructions. Poly $(A)^+$ RNA was subsequently purified from the isolated total RNA using Oligotex ™ mRNA purification kit (QIAGEN Inc., Chatsworth, CA, USA). First-strand cDNA was synthesized from the purified mRNA using SUPERSCRIPT™ II reverse transcriptase (GIBCO BRL®, Burlington, ON, Canada). The first-strand cDNA was synthesized by 3' rapid amplification of cDNA ends (3' RACE). Essentially, 1 μg of the purified poly $(A)^+$ RNA was added to 50 pmoles of oligo(dT) adapter primer: [5'-GACTCGAGTCGACATCG$(T)_{17}$-3'], the mixture was heated to 70 °C for 10 min and subsequently quick chilled on ice. The following was added to the cooled reaction

mixture: 1 µl of mixed dNTP stock containing 10 mM each dATP, dCTP, dGTP and dTTP (Amersham Pharmacia Biotech Inc., Baie de Urfe, Quebec) at neutral pH, 1 X first strand buffer (50 mM Tris-HCl pH 8.3/ 75 mM KCl/ 3 mM $MgCl_2$) and 2 µl of 0.1 M DTT. The mixture was subsequently heated to 42 °C for 2 mins followed by the addition of 200 units of SUPERSCRIPT™ II reverse transcriptase. The reaction was carried out at 42 °C for 50 min, followed by 70 °C for 15 min. The first-strand cDNA was used as the template for subsequent PCR amplifications. Prior to PCR amplifications, the cDNA was incubated with 5.0 units of RNase H at 37 °C for 2 mins to destroy potentially interfering RNA.

2.29. *PCR amplifications*

The strategy used for amplification of DNA encoding the protein described in chapter 4 is schematically outlined in Figure 2.1.

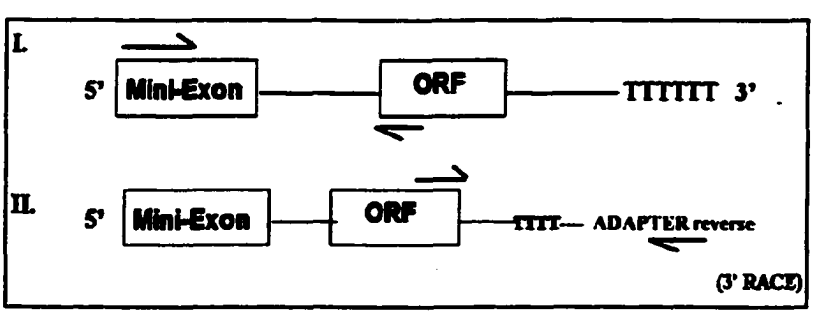


Figure 2.1. ***PCR-amplification strategy for DNA encoding T. simiae protein.***

The strategy relies on the highly conserved 39 nt RNA leader transcript (called the mini-exon) which occurs in every mature trypanosome mRNA. The upstream non-coding region and the 5' coding regions of the *T. simiae*-specific open reading of interest were amplified using a 5'-*T. simiae* mini-exon [Sturm *et al.*, 1998], (forward) primer :

[5'-ATTATATTACAGTTTCTGTACTAT-3'] and a 3'-internal (reverse) primer: [5'-TCTTGAGCTCGTTGTAGT-3'] that was specific to the coding sequence corresponding to the *T. simiae* amino acid sequence of interest. The PCR reaction mixture (25 µl total volume) contained 25 pmoles each of the forward and reverse primers, 1 X *Taq* DNA polymerase buffer (100mM Tris-HCl pH 9.0/ 500 mM KCl/ 15 mM $MgCl_2$), 1 µl of mixed dNTP stock containing 10 mM each dATP, dCTP, dGTP and dTTP, 2 µl of the cDNA template (see above) and 2.5 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech Inc., Baie de Urfe, Quebec). The amplification cycle was: 20 cycles of 45 °C annealing (45 sec) and 72 °C extensions (2 min), followed by another 10 cycles at 50 °C annealing (45 sec) and 72 °C extensions (2 min) and a final extension at 72 °C for 10 min. The nucleotide sequence obtained from sequencing the amplified fragment was further used to design a *T. simiae* ORF specific (forward) primer: [5'-CTCGCCATGCAGTTTC-3'] that represented the 5' end of the coding region encompassing the ATG start codon. The coding and the downstream non-coding regions were amplified using the *T. simiae* ORF specific (forward) primer: [5'-CTCGCCATGCAGTTTC-3'] and a 3' adapter (reverse) primer: [5'- GACTCGAGTCGACATCG-3']. The PCR reaction mixture (25 µl total volume) was essentially the same as above and the amplification cycle was: 10 cycles of 45 °C annealing (45 sec) and 72 °C extensions (2 min), followed by another 25 cycles of 50 °C annealing (45 sec) and 72 °C extensions (2 min). The nucleotide sequences from both sets of PCR amplifications were aligned to obtain a consensus *T. simiae*-specific predicted open reading frame (ORF). This in turn was used to design ORF-specific forward and reverse primers that were designed from the adjacent upstream and

downstream non-coding regions. Finally, the entire coding region was amplified using an upstream ORF-specific (forward) primer [5'-GAAAATAAGGAAGGTCATTAGTGG-3'] and a downstream ORF-specific (reverse) primer: [5'-CGAAGTGGAACATTGAGGAGAC-3']. The PCR protocol used was: 35 cycles of 50 °C annealing (45 sec) and 72 °C extensions (2 min). All primers were designed using the primer design software, Primer Premier 5 (PREMIER Biosoft International, Palo Alto, CA, USA). The *Taq*-amplified PCR fragments were cloned into pCR-TOPO vectors by TOPO™ TA-cloning (Invitrogen ™ Canada Inc., Burlington, Ontario).

2.30. *Expression studies*

The protein encoded by the *T. simiae*-specific ORF was expressed as a recombinant protein using the pET-15b vector with an N-terminal six histidine fusion tag (Novagen Inc., Madison, WI, USA).

Forward and reverse primers were designed from the nucleotide sequence to amplify the coding region of interest from *T. simiae* cDNA. The ORF-specific (forward) primer [5'-TGGTGTCATATGATGGCGTCC-3'] introduced an *NdeI* restriction site at the ATG start codon (as underlined in the primer sequence) and the ORF-specific (reverse) primer [5'-CGCACGGATCCATCACGAGTCAA-3'] introduced a *BamHI* restriction site (the underlined sequence) downstream of the ORF. The PCR reaction mixture (25 µl total volume) contained 25 pmoles each of the forward and reverse primers, 1 X *Pfu Turbo*® DNA polymerase buffer (20 mM Tris-HCl pH 8.8/ 10 mM KCl/ 10 mM $(NH_4)_2SO_4$/ 2 mM $MgSO_4$/ 0.1% Triton® X100/ 0.1 mg ml^{-1} nuclease-free BSA), 1 µl of mixed dNTP stock containing 10 mM each dATP, dCTP, dGTP and dTTP, 2 µl of

the cDNA template (see above) and 2.5 units of *Pfu Turbo®* DNA polymerase (Stratagene Cloning Systems, La Jolla, CA, USA). A 'touch-down' PCR reaction was performed: 3 cycles of amplifications each was performed with temperatures decreasing by 1 °C starting at 60 °C annealing (45 sec) and 72 °C extensions (2min) to a final temperature of 56 °C annealing (45 sec) and 72 °C extensions (2min), followed by another 10 cycles at 55 °C annealing (45 sec) and 72 °C extensions (2 min). The amplification product was digested with *NdeI* and *BamHI* restriction enzymes and the resulting fragment was ligated to a similarly digested pET-15b expression vector using T4 DNA Ligase (GIBCO BRL®, Burlington, ON, Canada). The nucleotide sequence of the pET-15b-*T. simiae*-specific ORF construct was determined prior to attempted expression of the coding region to verify whether the putative sequence was in the correct reading frame. Subsequently, the construct was transformed into the *E. coli* expression strain BL21(DE3) and overexpression was attempted with varying concentrations of IPTG (0.4 mM to 1.5 mM). Another set of expression studies was carried out using co-transformation of the pET-15b-*T. simiae*-specific ORF with a temperature sensitive vector, pGP-1 (that has a copy of RNA polymerase), into the *E. coli* expression strain BL21. Expression was attempted by heat shock at 42 °C after growing the cells to a density of 0.6 – 0.8 at OD_{600}. Controls were performed using the pET-15b vector alone and the harvested cell pellets were analysed by SDS-PAGE.

3. Biochemical and immunological characterisation of surface molecules of *Trypanosoma simiae* and *Trypanosoma congolense* procyclic culture forms.

When BSF of African trypanosomes are ingested by tsetse flies, they differentiate into procyclic forms in the fly midgut [Vickerman *et al.*, 1988]. This transformation results in the loss of the VSG surface coat and expression of the immunodominant, stage-specific surface glycoproteins, the procyclins [Roditi *et al.*, 1987; Richardson *et al.*, 1988; Mowatt *et al.*, 1989; Roditi *et al.*, 1998]. The vast majority of research on procyclins has involved *T. brucei* spp. and has shown that these surface molecules are immunologically and biochemically distinct from those of other species of trypanosomes, including members of the subgenus *Nannomonas*. To obtain possible clues to procyclin function, it is important to study other trypanosome species. Here I outline studies on the surface molecules of *T. simiae* and *T. congolense*. These species, though both members of the same subgenus (*Nannomonas*), show different host ranges. *T. congolense* is infective for almost all domestic animals whereas *T. simiae* is almost exclusively a specific parasite of pigs. Both *T. congolense* and *T. simiae* can be transmitted by diverse species of *Glossina*. Metacyclic forms of these parasites (the subgenus *Nannomonas* also contains *T. godfreyi*) develop in the proboscis of tsetse. In contrast, members of the subgenus *Trypanozoon* (*T. brucei* spp.) develop in the salivary glands [Mulligan, 1970]. Since parasites of the two subgenera express different procyclins [Stebeck and Pearson, 1994], it is tempting to suggest that tissue tropism is influenced by these surface disposed molecules. If this is the case, then some features should be shared between species of the same subgenus. To examine this hypothesis, I set out to study the procyclins of *T. simiae* and *T. congolense*.

My project focused on a comparative study of the surface molecules of procyclic culture forms (PCF). Despite their different host ranges, *T. simiae* and *T. congolense* have the same developmental cycles in tsetse and thus may share certain features that within their life cycle allow similar interactions within the fly.

3.1. *Analysis of T. congolense and T. simiae proteomes.*

High resolution, two-dimensional gel autoradiograph patterns of ^{35}S-methionine labelled proteins of *T. simiae* and *T. congolense* PCF are shown in Figure 3.1. Melanie 3, 2-D analysis software, was used to compare the gel spot patterns. A separate figure, (Figure 3.2) shows a superimposed profile of the 2-D patterns. The *T. simiae* gel spots are indicated by red '+' (this gel autoradiograph is visible) and the *T. congolense* gel spots are outlined by blue lines. Even though several major spot constellations were identical and were used as reference constellations by the Melanie 3 software, a gel automatch function was unable to superimpose the other protein spots. The composite image reveals that most of the labelled proteins migrated to different positions, indicating that the majority of the *T. simiae* and *T. congolense* labelled proteins are different from each other. Thus, the 2-D gel electrophoresis results showed that *T. congolense* Kilifi clone K45/1 and *T. simiae* CP-11 procyclic proteomes were drastically different. This is a bit of a surprise since these two species are morphologically indistinguishable, are members of the same subgenus and have the same developmental cycle. Nevertheless, these parasites may have evolved separately over many millions of years [Overath *et al.*, 2001] and thus may exhibit quite divergent protein compositions. However, it is possible that if the encoding genes of these two species were available in the database and their

sequences could be compared, the majority of the polypeptide sequences would probably be similar. The different proteome profiles may be due to different posttranslational modifications of the same polypeptide or due to peptide microheterogeneity exhibited by these two species. Such modifications could alter the total charge of the proteins or their molecular masses and this could alter their migration positions. It should be pointed out that many proteins may not contain methionine and thus would not be detected with this 2-D technique.

Figure 3.1. ***2-D gel autoradiograms of ^{35}S-methionine-labelled T. simiae and T. congolense proteins.***

A: T. simiae CP11. B: T. congolense K45/1. The overlapping spot constellations used as references are outlined by boxes. These reference spot clusters were recognised both visually, by overlapping the autoradiograms on a light box, and by Melanie 3 two-dimensional gel analysis software. The apparent molecular masses (in kiloDaltons) are indicated on the right-hand edge of the composite image.

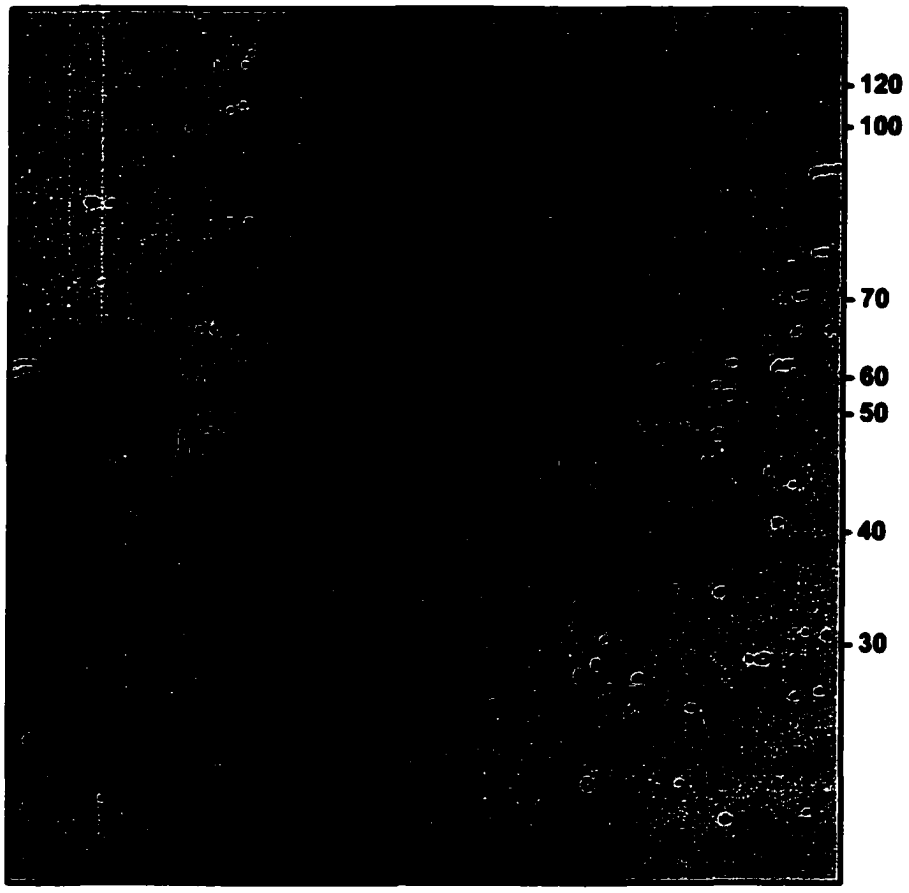


Figure 3.2. ***Superimposed, composite 2-D gel autoradiograms of ^{35}S-methionine-labelled T. simiae and T. congolense proteins.***

T. simiae CP11 gel spots are indicated by red '+'. T. congolense K45/1 gel spots are represented by blue outlines. The reference spot constellations were recognised both visually, by overlapping the autoradiograms on a light box, and by Melanie 3 two-dimensional gel analysis software. The apparent molecular masses (in kiloDaltons) are indicated on the right-hand edge of the composite image.

A comparison of 2-D gel profiles of *T. brucei* spp. and *T. congolense* has previously shown that the proteomes of procyclic forms of these different species (from different subgenera) are remarkably different [Anderson *et al.*, 1985]. Although *T. simiae* and *T. congolense* are both members of the same subgenus, the drastically different proteome profiles reported here raise the possibility that the procyclin analogues of the parasites of the subgenus *Nannomonas* may have different polypeptide moieties. Unfortunately, there is no methionine present in the GARP sequence (the only known procyclin in *T. congolense*), hence it was not visible in the 2-D gel autoradiograph profile. It is also not known if any procyclins of *T. simiae* contain methionine. As the GARP gene has been previously sequenced [Bayne *et al.*, 1993] and the sequence is available in the database, the next logical step was to determine whether the GARP gene was present in the *T. simiae* CP11 genome.

3.2. *Southern blot probing of the GARP gene in the subgenus Nannomonas.*

In-gel Southern blot analysis was performed by hybridization of a *T. congolense* GARP probe to Bam HI-digested genomic DNA of *T. simiae, T. congolense* and *T. b. brucei* PCF to determine if DNA sequences encoding the *T. congolense* GARP procyclin were present in *T. simiae*. The results of one of the experiments performed are shown in Figure 3.3. No hybridization was seen with digested genomic DNA from *T. simiae* (lane 1) or from *T. b. brucei* (lane 3; negative control), even with the low – moderate stringency conditions used (0.25 M Na_2HPO_4, pH 7.2/ 7% SDS). As expected, a single band of approximately 700 bp was detected with the genomic DNA from *T. congolense* (lane 2; positive control).

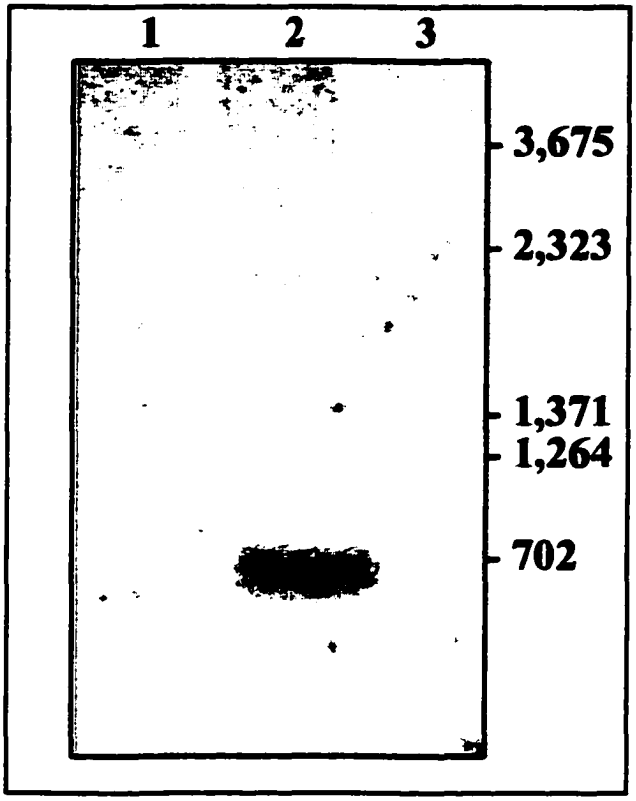


Figure 3.3. ***Southern blot analysis of Bam-HI-digested genomic DNA of procyclic trypanosomes probed with GARP DNA from T. congolense K45/1.***

Lane 1: T. simiae CP11. Lane 2: T. congolense K45/1. Lane 3: T. brucei 427.01. Molecular size standards (in base pairs) are shown on the right hand side of the autoradiogram.

Although the genes encoding GARP are known to be conserved among *T. congolense* Savannah, Forest and Kilifi subgroups of the parasites [Asbeck *et al.*, 2000], the GARP gene was previously reported as absent from one clone of *T. simiae* [Garside and Gibson, 1995]. My results with a different clone, *T. simiae* CP11, further confirm the possibility that the GARP polypeptide may not be expressed in procyclic

forms of *T. simiae*. Since it is likely that all procyclic trypanosomes express procyclin-like molecules, our results led us to investigate the surface molecules of PCF of *T. simiae* and *T. congolense* in order to compare them. My hypothesis was that these two species will express similar surface molecules that would allow them to interact with molecules of the tsetse vector and influence differentiation and tropism for different compartments within the fly.

3.3. *Analysis of surface molecules of procyclic trypanosomes using monoclonal antibodies.*

Surface directed mAbs raised against *T. congolense* PCF have been previously described [Beecroft *et al.*, 1993]. I analysed, by immunoflorescence microscopy and flow cytometry, one of these mAbs (TC 491) and a new mAb (TS 126) raised against *T. simiae* CP11. The immunofluorescence results using mAb TS 126 are shown in Figure 3.4. MAb TS 126 and mAb TC 491 both reacted strongly with the surface of *T. simiae* and *T. congolense* PCF, and did not react with *T. brucei* PCF (negative control). This result was surprising as GARP was shown to be absent from *T. simiae* CP11 and mAb TC 491 recognizes an epitope on GARP [Beecroft *et al.*, 1993]. This indicates that either the surface-directed, cross-reactive mAbs react with a protein that is antigenically similar to GARP (despite having a different amino acid sequence) or that the mAbs are directed against non-polypeptide epitopes that are shared between the PCF of these two parasites.

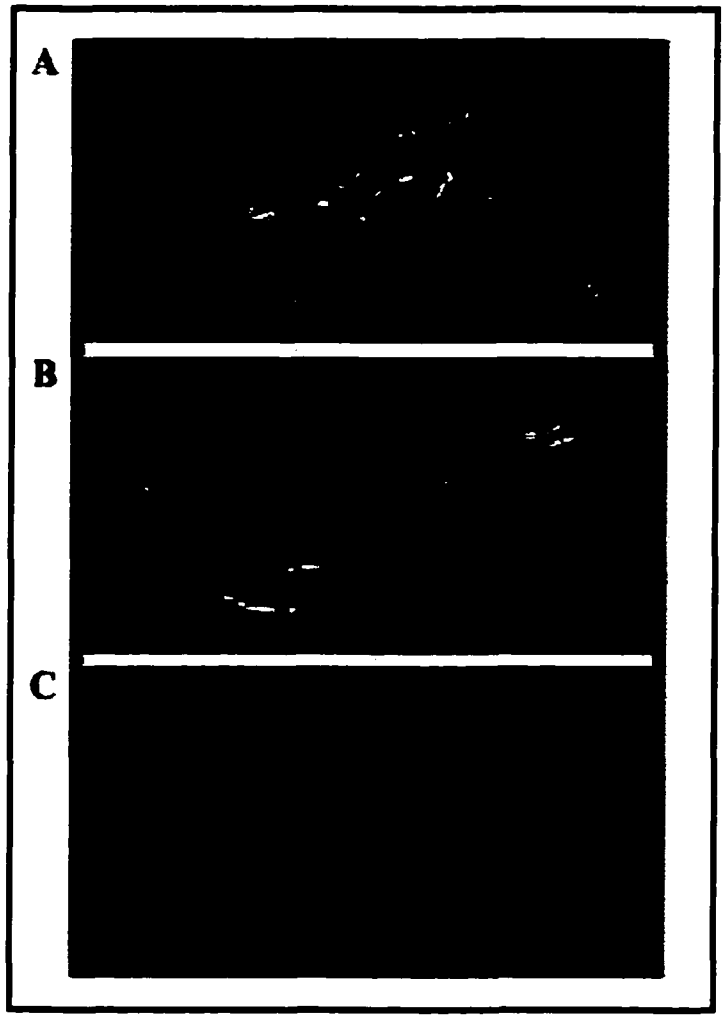


Figure 3.4. ***Immunofluorescence using surface directed mAb TS 126 on PCF trypanosomes.***

A: T. simiae CP11. B: T. congolense K45/1. C: T. brucei 427.01.

To further determine the specificity of the reactive mAbs, surface molecules from PCF of *T. simiae* and *T. congolense* were purified by reverse-phase high performance liquid chromatography (HPLC) using a procedure modified from that originally described for purification of procyclins from *T. brucei* spp. This procedure employs a semi-preparative octyl-Sepharose reverse-phase HPLC column [Ferguson *et*

al., 1993]. Column fractions were screened by indirect ELISA using two surface-binding mAbs (anti-*T. simiae* CP11 mAb TS 126 and anti-*T. congolense* K45/1 mAb TC 491) and a mixture of two mAbs that recognise membrane bound KMP-11 (mAbs L98 and L157). The ELISA profiles obtained are shown in Figure 3.5. These profiles are representative of those obtained in more than 10 experiments. Molecules detected with mAbs specific for the surface of the parasites eluted from lysates of both *T. simiae* and *T. congolense* between fractions # 45 and # 75, corresponding to a range of 38% propanol to 56% propanol. This rather late elution suggests that the molecules have hydrophobic properties that bind them to the column matrix. It is clear that the HPLC-eluted molecules from both species cross-reacted with both of the surface-directed mAbs. The KMP-11 membrane molecules, which are membrane-bound but not surface-disposed, eluted slightly before the major immunoreactive peak from *T. simiae* and at the beginning of the major antigen peak from *T. congolense*. This observation is reminiscent of the results obtained with *T. brucei*, where KMP-11 eluted just at the beginning of the procyclin peak [Stebeck *et al.*, 1996]. The surface mAb-immunoreactive HPLC fractions (minus those containing KMP-11) were pooled. Amino acid analysis of the proteins in the pooled HPLC peaks revealed much higher levels of amino acids in the *T. congolense* fraction when compared to *T. simiae* (data not shown). The pooled material was stored frozen until used for further biochemical and immunochemical analysis.

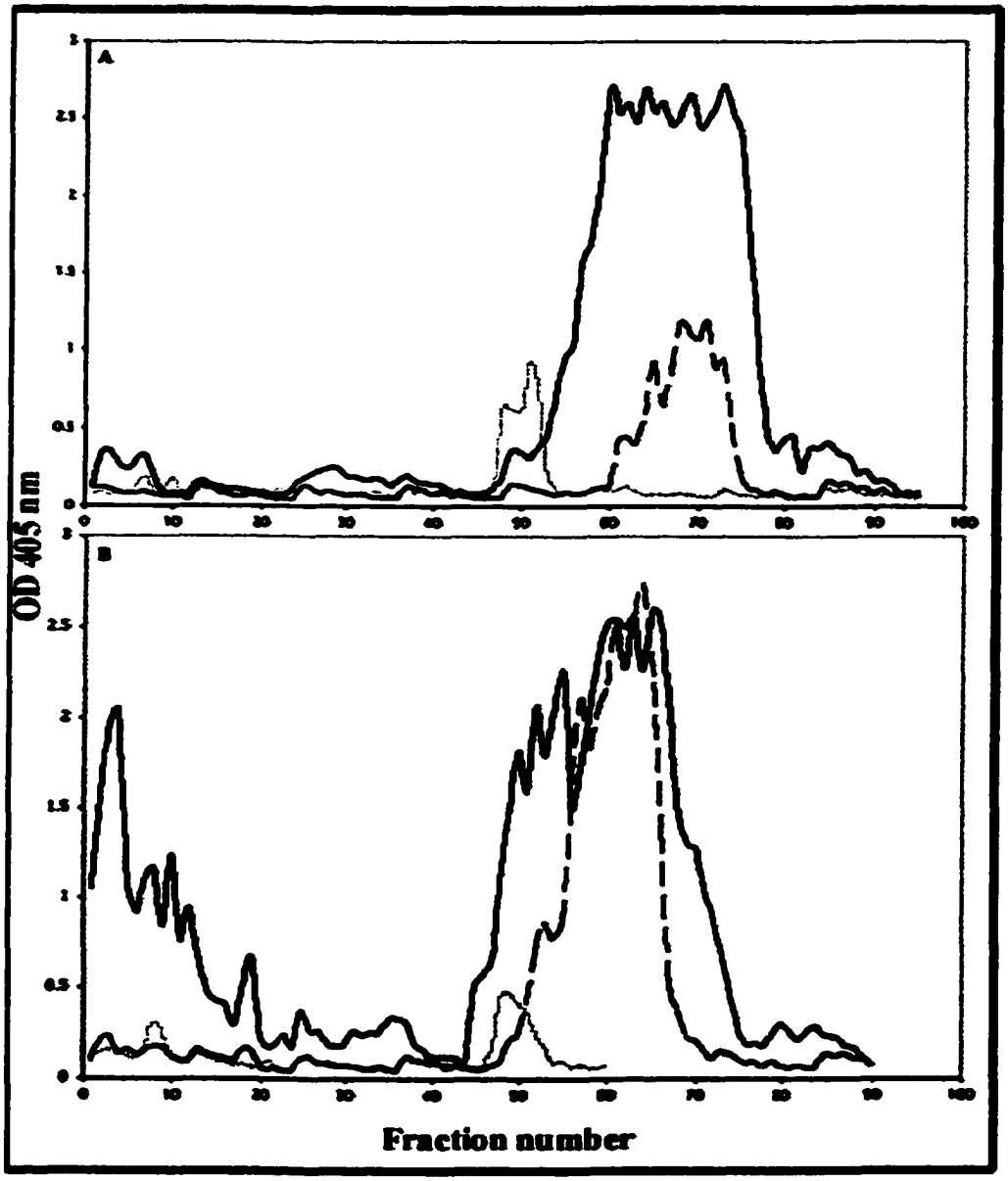


Figure 3.5. ***ELISA profile of T. simiae CP11 and T. congolense K45/1 PCF fractions eluted from a semi-preparative octyl-Sepharose HPLC column.***

A: T. simiae CP11. B: T. congolense K45/1. The primary mAbs were: TS 126 [—[, TC 491 [- - -[and L98/ L157 mixture [.....[.

3.4. *Acrylamide gel analysis of the HPLC-purified surface molecules of T. simiae and T. congolense PCF.*

HPLC-purified immunoreactive molecules from *T. simiae* and *T. congolense* were analysed using 10% SDS-PAGE gels. Representative stained gels are shown in Figure 3.6. Staining with GelCode Blue® (Figure 3.6, panel A) showed a major broad band at approximately 58 - 68 kDa for *T. simiae* (lane 1) and a broad doublet at approximately 45 - 56 kDa for *T. congolense* (lane 2). Stains-All™ (Figure 3.6, panel B) revealed a broad band of approximately 55 - 65 kDa for *T. simiae* (lane 1) and another broad band centered at approximately 50 kDa for *T. congolense* (lane 2). Lower, broad smears were also seen running from 25 - 30 kDa (*T. simiae*; lane 1) and 22 – 42 kDa for *T. congolense* (lane 2). It is interesting that in all cases, the bands observed with both GelCode Blue® and Stains-All™ were broad smears and were shifted lower in *T. congolense* when compared to *T. simiae*. The bands detected by Stains-All™ were blue, suggesting that the molecules may be phosphorylated. Silver staining (Figure 3.6, panel C) revealed a faint dark band at 66 kDa and a broad clear area centered at 30 kDa for *T. simiae* (lane 1). A dark, fairly sharp band at approximately 68 kDa, a broad very dark band centered at 46 kDa and a clear area between 20 kDa and 42 kDa were visible with *T. congolense* (lane 2). Here it is interesting that a thin, sharp, dark band was observed at 28 kDa, in the middle of the clear area. Since non-glycosylated GARP has a predicted mass of 28 kDa [Bayne *et al.*, 1993], the band may be this polypeptide. Thus, the SDS-PAGE profiles of the HPLC-purified molecules revealed that there are at least two major species of surface

molecules expressed by each parasite. In addition, the SDS-PAGE profiles suggested that the surface molecules of *T. simiae* and *T. congolense* PCF, although broadly similar, show differences in gel mobility and staining characteristics and are thus likely to be biochemically distinct.

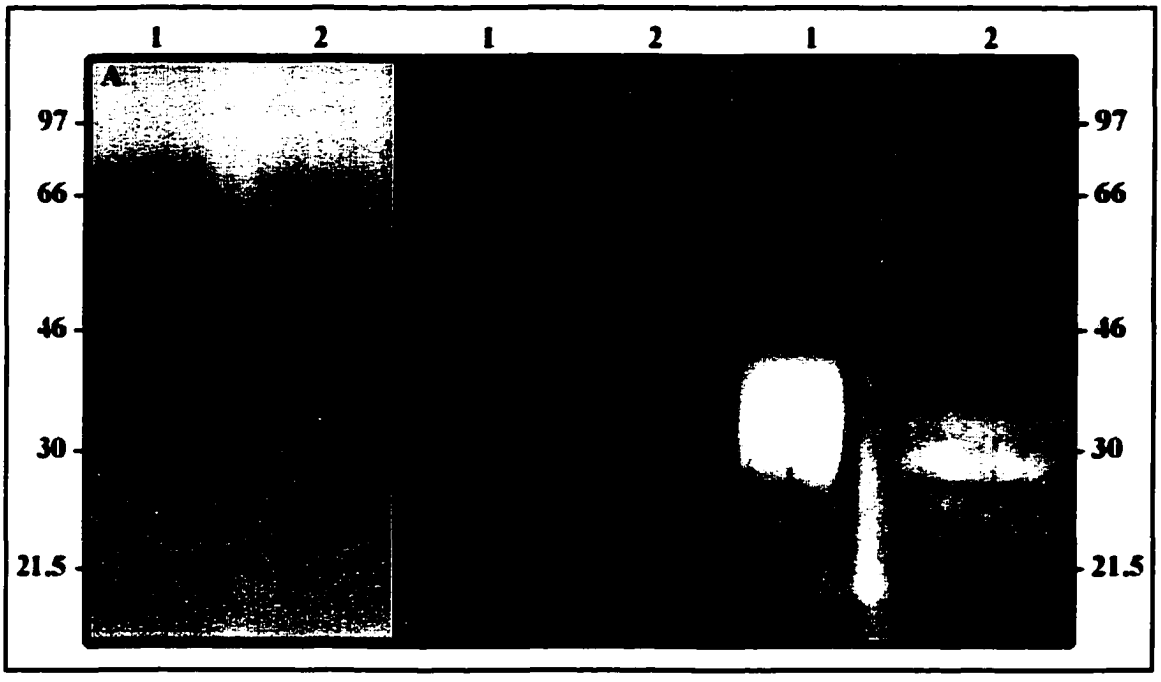


Figure 3.6. ***SDS-PAGE analysis of reverse-phase HPLC-purified molecules from PCF of T. simiae CP11 and T. congolense K45/1.***

A: GelCode® Blue stain. B: Stains-All™. C: Silver stain.
Lane 1: T. simiae CP11 PCF. Lane 2: T. congolense K45/1 PCF.
The apparent molecular masses are shown in kiloDaltons on the right hand side of the figure.

3.5. *Expression of GARP in T. congolense and T. simiae PCF*

Immunochemical approaches, high resolution 2-D gel electrophoresis and mass spectrometry were used in an attempt to identify GARP polypeptide sequences in the HPLC-purified surface molecules from both *T. simiae* and *T. congolense.* To detect the immunoreactive GARP polypeptide, immunoblots were performed on the HPLC-purified molecules from both species of PCF using a rabbit antiserum derived against *T. congolense* GARP that had been deglycosylated using anhydrous TFMSA. The results are shown in Figure 3.7. No immunoreactivity was seen with HPLC-purified molecules of *T. simiae* (lane 1) or *T. brucei* (negative control; lane 3). However, a dark broad band centered at 46 kDa and another sharp band at approximately 28 kDa were seen with molecules from *T. congolense* (lane 2). The 46 kDa broad band corresponds to the glycosylated form of GARP, whereas the 28 kDa band could be the non-glycosylated GARP polypeptide, as it corresponds to its predicted mass [Bayne *et al.*, 1993].

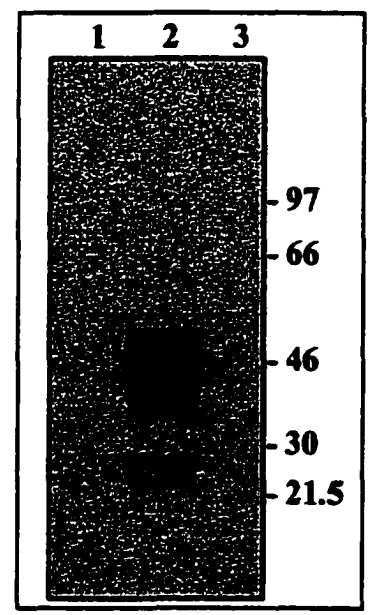


Figure 3.7. ***Immunoblot analysis of HPLC-purified molecules of T. simiae CP11, T. congolense K45/1 and T. brucei 427.01 PCF using an antiserum specific for deglycosylated GARP polypeptide.***

Rabbit anti-TFMSA treated T. congolense GARP was used as the primary antibody.

Lane 1: T. simiae CP11 HPLC-purified molecules.
Lane 2: T. congolense K45/1 HPLC-purified molecules.
Lane 3: T. brucei 427.01 HPLC-purified molecules.
Apparent molecular masses are shown in kiloDaltons on the right-hand of the figure.

Since epitopes could be denatured during SDS-PAGE and thereby perhaps not be detected in *T. simiae* by immunoblotting, another sensitive method was used for detection of GARP polypeptide. Electrospray ionization (nanospray)-quadrupole time-of-flight (ESI-QTOF) mass spectrometry was used to sequence tryptic peptides of the HPLC-purified parasite molecules after their separation by SDS-PAGE. Prior to mass spectroscopic analysis, staining of SDS-PAGE gels was performed using highly sensitive colloidal Coomassie Brilliant Blue G-250. Amino acid analysis of the Coomassie-stained bands from *T. simiae* showed low levels of amino acids in each. However, several attempts at N-terminal sequencing of the proteins in each of these bands failed. The Coomassie blue staining patterns seen with both *T. simiae* and *T. congolense* purified molecules were similar to those obtained with Stains-All™ (see Figure 3.6, B). These bands and a broad band at the dye front were cut and processed for mass spectroscopy. Figure 3.8, panel A, shows the tryptic peptide survey scan of the major 46 kDa band from SDS-PAGE separated, HPLC-purified *T. congolense* molecules. Figure 3.8, panel B shows the tandem mass spectrum of ions produced by fragmentation of the peptide of mass 530.2651 (indicated by arrows). The amino acid sequence of the peptide obtained by searching the database with the peptide fragmentation data using the Mascot MS/MS Ions Search algorithm (Matrix Science; London, UK: http://www.matrixscience.com/) was: GVDVATEAAAR, which perfectly matched amino acids 94 – 103 of the GARP sequence [Bayne *et al.*, 1993]. In fact, the only database hit obtained was GARP. Several other peptides were fragmented and the sequences also matched sequences of GARP (not shown), yielding unequivocal identification of the *T. congolense* procyclin. Mass spectroscopic analysis of several Coomassie Blue stained protein bands from

T. simiae HPLC-purified material did not reveal any polypeptide sequence related to GARP. This experiment was repeated with several batches of HPLC-purified material from *T. simiae* with the same results, clearly indicating that the GARP protein was not expressed on the surface of *T. simiae* PCF. This result further validated the Southern blot analysis (discussed earlier), where it was shown that the GARP gene was absent from *T. simiae*.

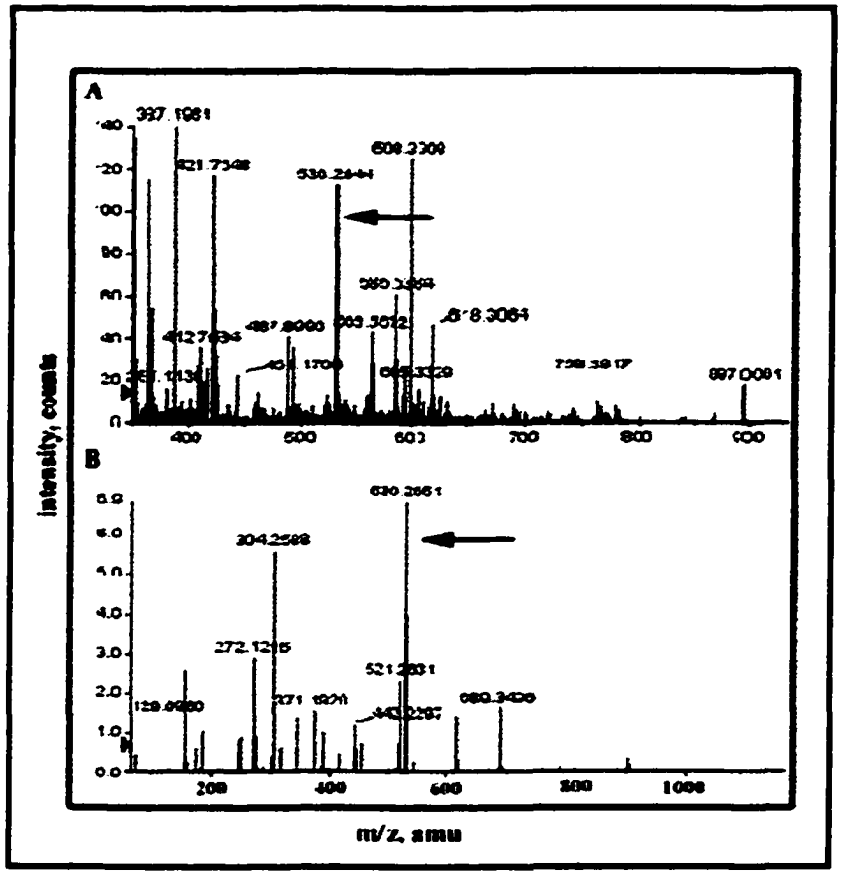


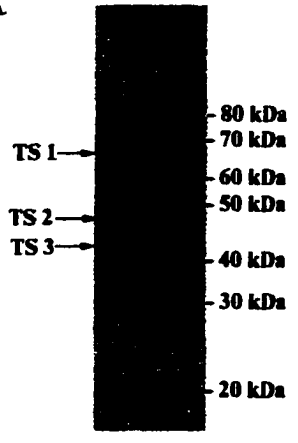
Figure 3.8. ***Tandem nanospray MS/MS analysis of trypsin-digested peptides from HPLC-purified molecules of T. congolense separated by 1-dimensional SDS-PAGE.***

Nano-electrospray ionization (ESI) was used to introduce ions into a PE-SCIEX Q-STRi quadrupole time-of-flight mass spectrometer.

A: Survey scan of all peptides generated. B: Fragmentation of the selected peptide. The sequence calls and b-series ions are indicated on the spectrum. The peptide sequence, GVDVATEAAAR, was identified as amino acid positions 94 to 103 of GARP.

Sequencing of several peptides from *T. simiae* by Q-TOF mass spectrometry, did however, reveal several unique sequences (Table 3.1), none of which matched any sequence in public databases. This result further confirmed the absence of GARP from *T. simiae*. The results led us to investigate the biochemical characteristics of the cross-reacting epitopes from the purified molecules of *T. simiae* and *T. congolense* PCF. The question asked at this point was: are the crossreacting, surface disposed epitopes expressed polypeptide or carbohydrate?

Table 3.1. ***Peptide sequences obtained by tandem nanospray MS/MS analysis of trypsin-digested peptides from HPLC-purified molecules of T. simiae separated by 1-dimensional SDS-PAGE.***

A	B. Sample	C. Mass	D. Peptide sequence
	TS 1	453.2	1. AQTEGPFR
	TS 1	544.7	2. SNQQNFHSK
	TS 1	668.3	3. AQLAASQNDDFR
	TS 1	751.8	4. QWAGTPEAEWATR
	TS 2	450.2	5. EAPLGLTAK
	TS 2	666.3	6. TFWVVEQLPGR
	TS 2	707.8	7. SNLDSSAVATFFR
	TS 2	426.3	8. LASGGGHPR
	TS 2	614.3	9. LPWVEPSDER
	TS 3	617.82	10. DTDGPFSVQLR
	TS 3	549.8	11. HNAFVTLAAR

A: HPLC-purified molecules of *T. simiae* separated by 1-dimensional SDS-PAGE and stained with colloidal Coomassie Brilliant Blue G-250.

The bands used for generation of tryptic peptides are indicated by the arrows to the right of the figure.

3.6. *Analysis of the crossreacting epitopes of T. simiae and T. congolense PCF*

In order to examine whether or not the HPLC-purified molecules from PCF of *T. simiae* and *T. congolense* were glycosylated, SDS-PAGE separated molecules on blots were oxidized with sodium meta-periodate, which oxidizes the vicinal hydroxyls of the carbohydrates to aldehydes. These were then biotinylated by reaction with biotin hydrazide [O'Shannessey *et al.*, 1987] and the biotin-labelled molecules were detected with streptavidin-horseradish peroxidase and chemiluminescence substrate. The results are shown in Figure 3.9. Two major glycosylated bands were observed in the butanol extracts of the delipidated cell pellets from both parasites. With *T. simiae,* there was a broad, very dark smear at approximately 23 kDa to 88 kDa (lane 1). With *T. congolense,* a less dark smear was seen from 25 kDa to 88 kDa. This smear showed darker bands centered at approximately 30 kDa and 66 kDa (lane 2). Therefore, these results showed that the two major broad bands that were detected in each species were heavily labelled by treatment with biotin hydrazide, indicating that they contained carbohydrates.

The procyclins from *T. brucei* spp. and GARP from *T. congolense* have been shown to be glycosylated (for review see chapter 1). It has been hypothesised that the procyclins form a glycocalyx covering the parasite membrane and protect the surface of the procyclics from proteases in the tsetse midgut. Thus, it is not surprising that the surface molecules isolated from the PCF of *T. simiae* and *T. congolense* were found to be heavily glycosylated, similar to several of the procyclins of *T. brucei* spp.

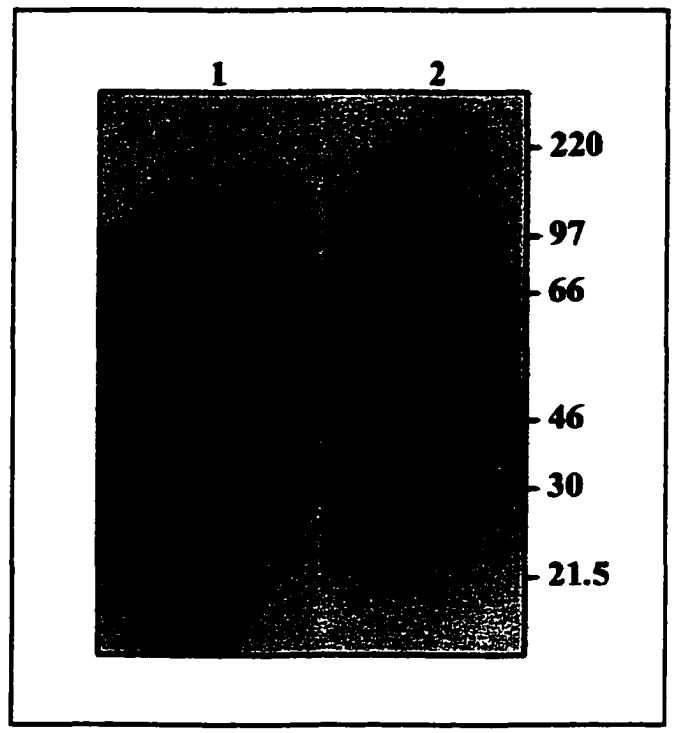


Figure 3.9. ***Detection of biotin-labelled carbohydrates on blotted, SDS-PAGE separated reverse-phase HPLC-purified molecules from T. simiae CP11 and T. congolense K45/1.***

Lane 1: T. simiae CP11 PCF. Lane 2: T. congolense K45/1 PCF.
Apparent molecular masses are shown in kiloDaltons on the right-hand side of the figure.

Immunoblots on the HPLC-purified molecules from *T. simiae* and *T. congolense* PCF were performed using the surface–directed mAbs TC 491 and TS 126 as primary antibodies. The results are shown in Figure 3.10. A faint broad band centered at approximately 55 kDa and a broad band between approximately 25 kDa and 45 kDa were seen with anti-*T. simiae* mAb TS 126 on *T. simiae* molecules (Figure 3.10, panel A, lane 1). Similar broad bands, shifted slightly lower, were seen with molecules from *T. congolense* (lane 2). This profile was observed repeatedly in more than 10 immunoblot experiments. Immunoblots performed using anti-*T. congolense* mAb TC

491 as the primary antibody (Figure 3.10, panel B) showed similar broad smears between 25 kDa and 40 kDa with both *T. simiae* (lane 1) and *T. congolense* molecules (lane 2). Broad bands centered at 66 and 55 kDa were also seen. Again the bands were shifted slightly lower in *T. congolense* when compared to those of *T. simiae*.

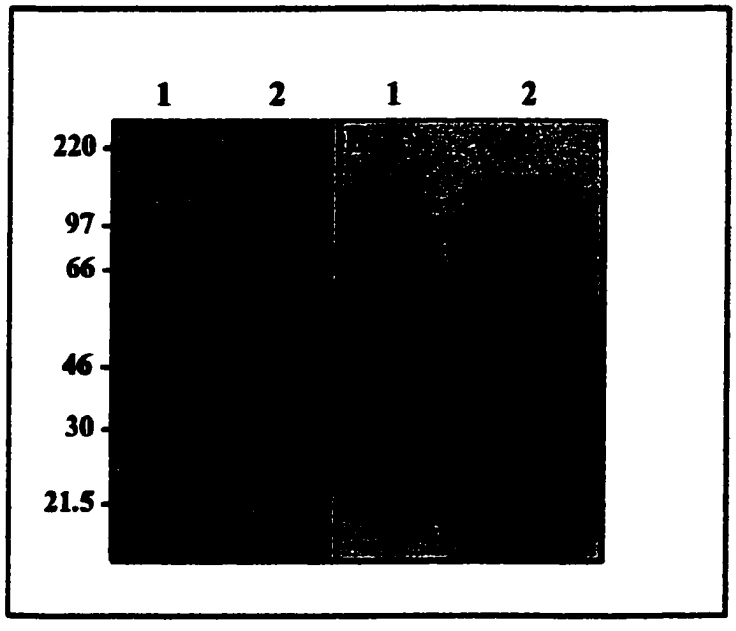


Figure 3.10. ***Immunoblot profile of HPLC-purified molecules of T. simiae CP11 and T. congolense K45/1 PCF.***

A: mAb TC 491 primary antibody. B: mAb TS 126 primary antibody.
Lane 1: T. simiae CP11. Lane 2: T. congolense K45/1.
The apparent molecular masses are shown in kiloDaltons on the left hand side of the figure.

The above two sets of results, taken together, indicate that the immunoreactive molecules are glycosylated. Finally, to unequivocally determine whether the carbohydrate moieties were the cross-reacting epitopes, several immunoblot experiments were performed after chemical modification of the surface molecules. Immunoblotting

was performed after deglycosylation of the HPLC-purified molecules with anhydrous trifluoromethane sulfonic acid (TFMSA) that destroys the glycosidic bonds while leaving the peptide bonds intact [Edge *et al.*, 1981]. The results are shown in Figure 3.11. TFMSA treatment abrogated binding of mAb TS126 (lanes 1 and 2). However, the expected immunoblot pattern was seen with HPLC-purified, non-deglycosylated molecules from *T. congolense* used as a positive control (lane 3). Identical results were obtained using mAb TC 491 (not shown). Therefore, the cross-reacting epitopes of *T. simiae* and *T. congolense* detected by these two surface-binding mAbs were carbohydrates.

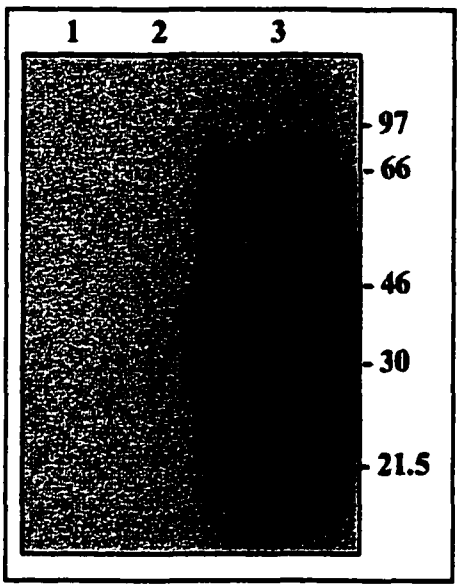


Figure 3.11. ***Immunoblot analysis of deglycosylated, reverse-phase HPLC-purified molecules of T. simiae CP11 and T. congolense K45/1 PCF.***

Anhydrous TFMSA was used to deglycosylate the HPLC-purified molecules. MAb TS 126 was used as the primary antibody.
Lane 1: T. simiae CP11. Lane 2: T. congolense K45/1. Lane 3: Control, non-deglycosylated T. congolense K45/1. Apparent molecular masses are shown in kiloDaltons on the right-hand side of the figure.

Treatment of the HPLC-purified molecules with PNGase F1 prior to immunoblotting did not alter the patterns obtained when compared to the patterns seen with untreated, purified molecules (not shown), indicating that the molecules from both *T. simiae* and *T. congolense* were not N-glycosylated. It is known that GARP, at least, does not have any N-glycosylation sites in the predicted amino acid sequence [Bayne *et al.*, 1993].

Thus it was shown that, despite the fact that GARP is not expressed in *T. simiae*, *T. congolense* and *T. simiae* PCF share surface-exposed carbohydrate epitopes. Taken together, the data suggest that the major HPLC-separated bands of *T. simiae* represent unique glycoproteins and that they share carbohydrate epitopes with each other, with GARP and with other surface molecules of *T. congolense*. However, the polypeptide sequences (if any) in *T. simiae* are different from the GARP sequence of *T. congolense*. The apparent high abundance of carbohydrates may explain the low yields of amino acids and the difficulty encountered in sequencing the purified surface molecules from the PCF of *T. simiae*, although simple N-terminal blockage could explain the latter. It is interesting that novel non-GARP, carbohydrate-containing, GPI-anchored surface molecules have recently been detected in *T. congolense* and that similar difficulties in obtaining protein sequences were encountered [Bütikofer et al., in press].

3.7. *Labelling of GPI-anchored molecules of T. simiae and T. congolense*

To determine whether the reverse-phase HPLC-purified molecules from *T. simiae* and *T. congolense* PCF were GPI-anchored (as shown previously for *T. brucei*

procyclins; see chapter 1), the parasites were biosynthetically labelled with [1-^{3}H] ethan-1-ol-2-amine hydrochloride. After an overnight labelling, the cell counts were between 1.0-1.25 x 10^8 for both *T. simiae* and *T. congolense* (observed in several experiments). This represents a doubling of cell numbers during the labelling period. In the experiment presented here, butanol extracts of the delipidated pellets contained 2.9 x 10^5 cpm (*T. simiae*) and 1.4 x 10^5 cpm (*T. congolense*), counts typical of that seen in several experiments. Butanol extracted, radiolabelled molecules were separated by SDS-PAGE and the gel was fluorographed. The results are shown in Figure 3.12. A broad band centered at 66 kDa and another broad band between 25 kDa and 45 kDa was seen with *T. simiae* (lane 1). A similar pattern was seen with *T. congolense*, although the bands were shifted slightly lower (lane 2). The incorporation of radioactivity was slightly higher in the band between 25 kDa and 45 kDa of *T. simiae* when compared to the band at 30 kDa for *T. congolense*.

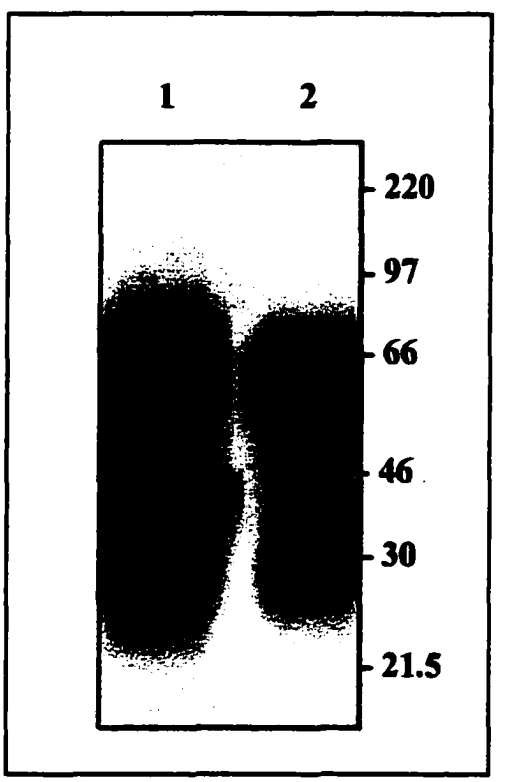


Figure 3.12. ***Fluorograph of SDS-PAGE separated, [1-^{3}H] ethan-1-ol-2-amine hydrochloride labelled, reverse-phase purified molecules from T. simiae CP11 and T. congolense K45/1.***

Lane 1: T. simiae CP11. Lane 2: T. congolense K45/1. Molecular masses are shown in kiloDaltons.

Thus, interestingly, in both species, the HPLC-purified molecules could be biosynthetically labelled with ^{3}H-ethanolamine. It is likely that they contained terminal ethanolamines that are involved in attachment of polypeptides to lipid anchors. Though non-protein linked GPI anchors such as free GPI lipids and lipophosphoglycans have been described in protozoan parasites, these structures lack ethanolamine. The hydrophobic properties revealed during the reverse-phase purification process of the surface molecules, together with the fact that they could be biosynthetically labelled with ^{3}H-ethanolamine clearly indicated that these glycoconjugates were GPI-anchored molecules. This was not surprising as the procyclins characterized from *T. brucei* spp. [Ferguson *et al.*, 1993] and GARP from *T. congolense* [Bütikofer *et al.*, in press] are both GPI-anchored. It is possible that all procyclins, regardless of trypanosome species, are GPI-anchored molecules, similar to the VSG surface molecules expressed in BSF [Ferguson *et al.*, 1993]. Thus, these parasites have surface disposed, immunodominant, GPI-anchored glycoconjugates expressed throughout their life cycle in both the mammalian hosts and in the insect vector.

Finally, the SDS-PAGE-separated, HPLC-purified molecules from *T. simiae* and *T. congolense* PCF were analysed for their ability to bind to proaerolysin, a toxin that is known to bind specifically to GPI anchors. The results are shown in Figure 3.13. No proaerolysin-binding molecules were detected in the HPLC-purified fractions from *T. simiae* PCF (lane 1), whereas a broad band of approximately 50 - 70 kDa was detected in *T. congolense* PCF (lane 2). This result was very interesting as it indicates that the GPI-anchors of *T. simiae* and *T. congolense* have different structures.

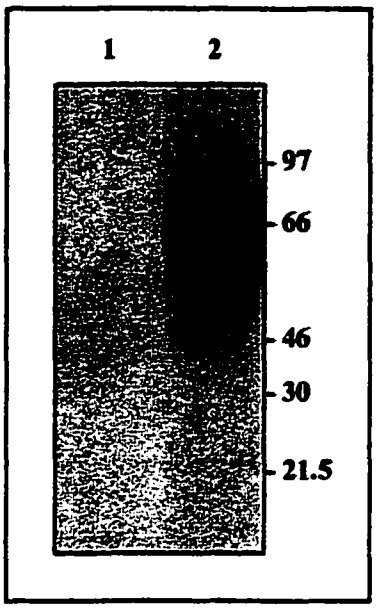


Figure 3.13. ***Detection of proaerolysin-binding molecules in SDS-PAGE- separated, HPLC- purified molecules from procyclic trypanosomes.***

Lane 1: T. simiae CP11 PCF. Lane 2: T. congolense K45/1 PCF. Apparent molecular masses are shown in kiloDaltons on the right-hand side of the figure.

It is of interest here that the procyclins characterized from *T. brucei* spp. do not bind to proaerolysin, whereas VSG does [Dr. J. T. Buckley, personal communication). Although the biochemistry of binding of this toxin is not understood in much detail, it is known that modification of the core structure of GPI-anchors by addition of sugars or phosphoethanolamine, or by acylation of inositol results in the loss of the binding [Nelson *et al.*, 1997]. Perhaps those kinds of modification are reflected in the differential binding to anchors of the two trypanosome species. What is clear that the anchor of at least one of the surface molecules is biochemically distinct from the others.

Summary

This study showed that there are several surface-disposed, major immunodominant molecules present in both *T. congolense* and *T. simiae* and that these are glycoconjugates. Southern blot analysis showed that the *T. simiae* CP11 PCF clone does not contain GARP genes. In addition, this clone does not express GARP polypeptide detected either immunologically or by mass spectrometry. However, *T. congolense* and *T. simiae* do share surface-exposed epitopes recognised by subgenus *Nannomonas*-specific mAbs and these epitopes are carbohydrates. These surface-disposed glycoconjugates, present in both *T. congolense* and *T. simiae*, are GPI-anchored. Although these membrane molecules are immunologically similar, there are biochemical differences between them as reflected in their different HPLC elution and gel staining profiles, the absence of GARP in *T. simiae* and differential binding of proaerolysin toxin. It seems plausible that the carbohydrate epitopes shared between these two species of the subgenus *Nannomonas* may be involved in interactions with the tsetse vector since both species of trypanosomes that express them share the same developmental cycle. If this is the case, then it appears that *T. congolense* and *T. simiae*, despite their widely different proteomes, have placed common carbohydrate structures on different carriers as a way to achieve the same function.

4. Identification of a novel gene product in *Trypanosoma simiae*

One of the objectives of my project was to identify and characterise surface disposed procyclins from *T. simiae* and *T. congolense* PCF. In the previous chapter, it was shown that several surface molecules of the PCF of *T. simiae* and *T. congolense* are GPI-anchored. It was interesting that GARP, the only procyclin glycoprotein previously identified in *T. congolense* was not present in *T. simiae*. Since the GARP gene was absent from the *T. simiae* genome and the protein was not expressed on the surface of the PCF of *T. simiae*, it is possible that *T. simiae* expresses procyclin polypeptide with sequences different than those of GARP. Many attempts to obtain N-terminal amino acid sequences from the HPLC-purified molecules from PCF of *T. simiae* were unsuccessful. Perhaps this was due to extensive glycosylation of the surface molecules that would hinder sequencing attempts. Alternatively, the proteins could have been blocked at their N-termini. Interestingly, similar attempts at identification and N-terminal sequencing of non-GARP surface molecules from PCF of *T. congolense* were also unsuccessful [Bütikofer *et al.*, in press]. Because of the difficulties encountered in obtaining sequence information from the various cell surface glycoconjugates of *T. simiae* PCF. I attempted sequencing of internal peptides after deglycosylation of these molecules. These could potentially hinder production of peptides and successful Edman degradation and amino acid sequencing.

4.1. *Sequencing of internal peptides from surface molecules of T. simiae PCF*

HPLC-purified molecules from *T. simiae* PCF were deglycosylated using anhydrous TFMSA, which destroys glycosidic bonds while leaving peptide bonds intact. Deglycosylated polypeptides were then cleaved using cyanogen bromide that cleaves at the carboxyl of methionine. Separation of the resulting CNBr peptides was initially attempted using Tricine-SDS-polyacrylamide peptide gel electrophoresis. However, the yields of peptides were very low and there was not enough peptide to be detected in the gels after staining with GelCode® Blue. This observation validated the amino acid microanalysis results obtained with the HPLC-purified surface molecules from *T. simiae* PCF (previous chapter). Here much higher levels of amino acids were seen in the *T. congolense* material when compared to the pooled peaks from *T. simiae*. The lack of peptides detectable in peptide gels also suggests that the surface molecules of the procyclic forms of *T. simiae* are comprised primarily of carbohydrates with the polypeptides being a minor component. Where polypeptides are involved, perhaps they may function only to attach the carbohydrates to the membrane. This hypothesis would also explain why the surface molecules of the procyclic forms of the two closely related species, *T. simiae* and *T. congolense*, appear to have unrelated polypeptide sequences yet have cross-reacting carbohydrate epitopes: the polypeptides would function as different "hangers" upon which are hung the same (carbohydrate) "coats".

To increase the sensitivity of peptide detection, CNBr peptides were also fractionated by reverse-phase HPLC using a microbore HPLC reverse-phase C_{18}, Ultrasphere ODS (5 μm), 2.0 mm X 25 cm column (Beckman Instruments Inc., Fullerton, CA, USA). This method is more sensitive than gel electrophoresis and can detect

picomoles of peptides. Whereas, microgram quantities of proteins are often required for detection by gel electrophoresis using standard staining methods. A profile of HPLC-separated CNBr peptides from one CNBr digest is shown in Figure 4.1. Peptides were detected by absorbance at 230 nm and gas-phase sequencing was attempted on several peaks. A 12 amino acid sequence (VDVDYNELKKFR) was obtained from one of the fractions (indicated by the arrow).

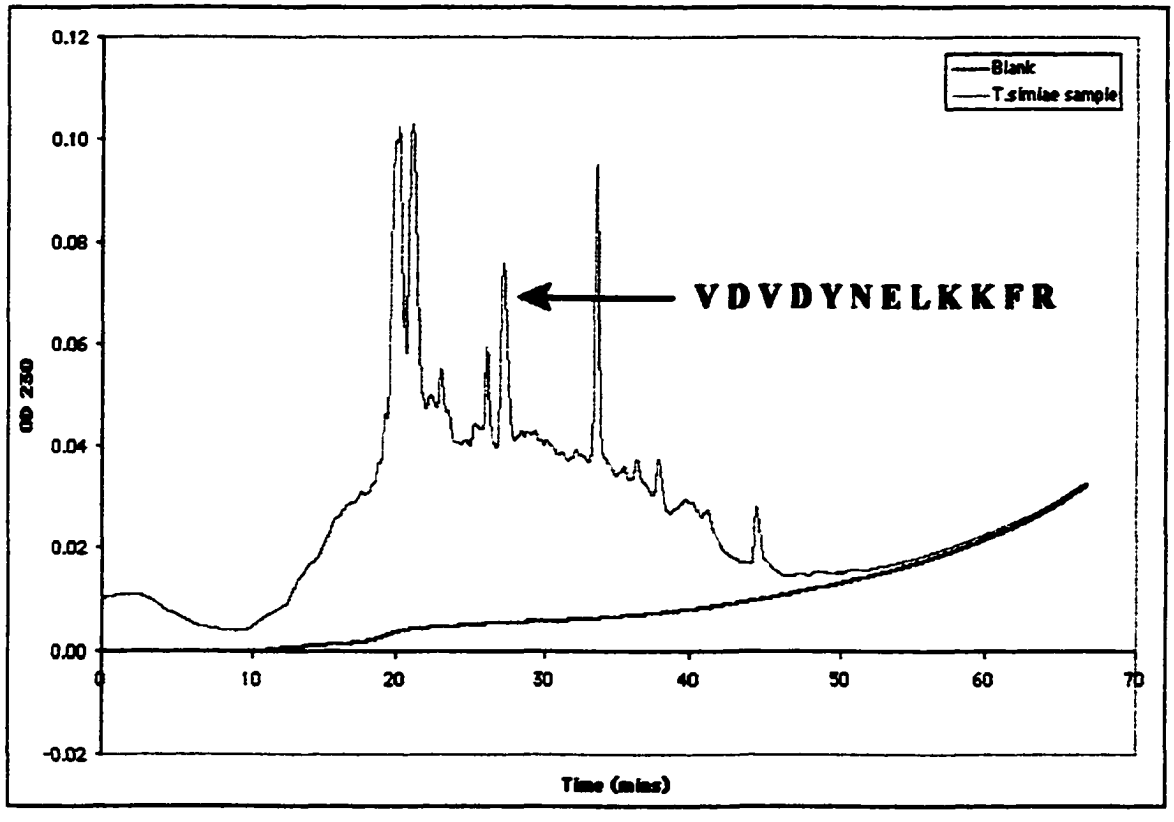


Figure 4.1. ***Reverse-phase HPLC profile of CNBr- peptides from HPLC-purified surface molecules of T. simiae PCF.***

A microbore (2mm) C18 column was used and peptides were detected by measuring absorbance at 230 nm. The amino acid sequence obtained from the peak indicated by the arrow.

4.2. *An unique open reading frame from T. simiae cDNA*

The amino acid sequence obtained with one of the *T. simiae* CNBr peptides was used to design a 3'-internal (reverse) degenerate primer, [5'-TCTTGAGCTCGTTGTAGT-3']. This reverse primer and a forward primer corresponding to the *T. simiae* mini-exon sequence were used for PCR amplification of *T. simiae* PCF cDNA in order to obtain the DNA encoding the protein (strategies employed for the PCR amplifications are explained in chapter 2). A 1056 bp ORF (TS ORF 1) was determined from the compiled sequence data from the PCR products and is shown in Figure 4.2. The corresponding polypeptide sequence translated from the DNA sequence is shown in Figure 4.3. These sequences (both DNA and protein) did not significantly match any existing sequences in the NCBI non-redundant database.

```
5'  ATG ATG GCG TCC CAC ACA TTG TCC GAT GCG CTC GCC ATG CAG TTT CAG TGG
CTA CAC GTT TTT GTT TTT CGG CGG CGT GAG CAC TAC ACG GTG GGT CGT CGG AGG
GCT CTC CTA TTT GCC GTT ATG CAC GCG TGT TTT CTG TTG GTG CTC TTT GGG TCC
TTC GTC AAC GAT ATT GTT TCC CTG TCA CTT GGG GTC CTG ATG CAG ACC GCC ACC
TTT GTC ATG TCC ATC ATG CAC GTG TGC GCC GTC ATG GAC TAC AGC GAG CGA TTG
GTA AAT GCG ATG GAA CTG GAA CAG CAG CTC AAT CCG TTA GTC ATC GCT GAG CTG
AGC GTT CGG TGC TTC GCT CAA ATG CAT TTT TTC ATG CTG CGG TGG TGG CTT ACG
TTT GCC ATT GGT GCG CTG GAG CTT TTC TAC AAC TAC TGG TTG TAC AGT CGG GGC
GTA CTT GTT GTC GAC GCC ACC ACC GCG TGG AGA CAG CTG GAG GCA CTG AAG GTG
GAT GGC AAG GTA CGC CTC GTC TTC CAA GGG GTT TTT CTT TTG GTG GTC ATC GCG
GAG CTT ATC TAT TTG ATG ATA ACG ATG TGA CAC AGA GCC GTT TTT GTC TCC TCA
ATG TTC CAC TTC GAA CAG ACT GAC TAT CTA TAT ATA TAT ATA TAT CTT TTC GAG
CGG CGT CAG TGC TTA CAT GGC TGC GTT AGT CCT TCA CGG TGC GTC GGA GTG CTG
CTA CGT TCG GAT GTT GGT GTG CGG CGG GGA CAT GGT TGA TGT GCC GTT GGA AAA
GGG GAG GGG AAG AGG CGT AAG TTA GGC ATC TTC TGT TTG CTG TGT GAC GCG TAT
TTG TGC GTA GTT GTT GTT CTT CCT TTA TTC GTT CAC ACC TCA CCA AAC CTT ATA
CGG GCG TCC CTC TGC GCC CTG TTG ATG AAT GGT TTT TGA GCG ACT GAG GGT GCT
GTT GGT GGT GGT GAT GAA TGG GTG GGT GGA TGG GGG GCC CGC GGG ATG TGG GTG
TTT TTA CTT CTT TCC TTC TTT AAA CTT TAT TTC GTG CTG GGG CTG CGT ACG CGC
ACG TGT GCT GCG GGG TGT GCT TTC CCG TGG TAA 3'
```

Figure 4.2. ***The DNA sequence (TS ORF 1) obtained by PCR amplifications of T. simiae cDNA.***

MMASHTLSDALAMQFQWLHVFVFRRREHYTVGRRRALLFAVMHACFLLVLFGSFV
NDIVSLSLGVLMQTATFVMSIMHVCAVMDYSERLVNAMELEQQLNPLVIAELSVRCFAQMH
FFMLRWWLTFAIGALELFYNYWLYSRGVLVVDATTAWRQLEALKVDGKVRLVFQGVFLLV
VIAELIYLMITMWHRAVFVSSMFHFEQTDYLYIYIYLFERRQCLHGCVSPSRCVGVLLRSDVG
VRRGHGWCAVGKGEGKRRKLGIFCLLCDAYLCVVVVLPLFVHTSPNLIRASLCALLMNGF
WATEGAVGGGDEWVGGWGARGMWVFLLLSFFKLYFVLGLRTRTCAAGCAFPW

Figure 4.3. ***The translated polypeptide sequence of TS ORF1 obtained from T. simiae cDNA.***

The underlined peptide sequence corresponds to the sequence used to design a synthetic peptide.

The exact amino acid sequence obtained from the microbore HPLC separation of the CNBr peptides that was used to design the peptide-specific primers was not seen in the translated polypeptide sequence. Perhaps microheterogeneity in sequences of different procyclin genes exists as with *T. brucei* spp. None of the several unique *T. simiae* – specific peptide sequences obtained by tandem nanospray MS/MS analysis of trypsin-digested peptides from HPLC-purified molecules (shown in Table 3.1), matched sequences in the translated polypeptide. However, the sequence obtained clearly represents an open reading frame. The question remained, is it expressed in PCF and if so where? The probability that TS ORF1 encodes a membrane - associated protein is high, as the amino acid sequence was obtained from the HPLC-enriched molecules purified from the procyclic forms of *T. simiae.*

I first attempted to express the ORF as a recombinant protein. Unfortunately, several attempts (over a six month period) at expression using various combinations of vectors and induction protocols in *E. coli* were not successful. Thus, another strategy was used for localisation of the polypeptide in the parasite. The predicted polypeptide sequence (Figure 4.3) was analysed for amino acid sequences that had potential to form

B-cell epitopes. The results of the protein analysis using Protean™, protein analysis software (DNAStar Inc., Madison, WI, USA) are shown in Figure 4.4. A nine amino acid peptide, RREHYTVGR, corresponding to amino acids 25 to 33 of the predicted polypeptide sequence (underlined in Figure 4.3) was predicted by the Doolittle, Karplus – Schultz, Wolf and Emini algorithms to be hydrophilic and flexible and have a high antigenic and surface probability index respectively, and thus should comprise a good B-cell epitope. A nine amino acid peptide was thus synthesised and was coupled to the carrier protein Imject® KLH. The coupled conjugate was used as the immunizing antigen to raise a mouse antiserum specific for the synthetic peptide.

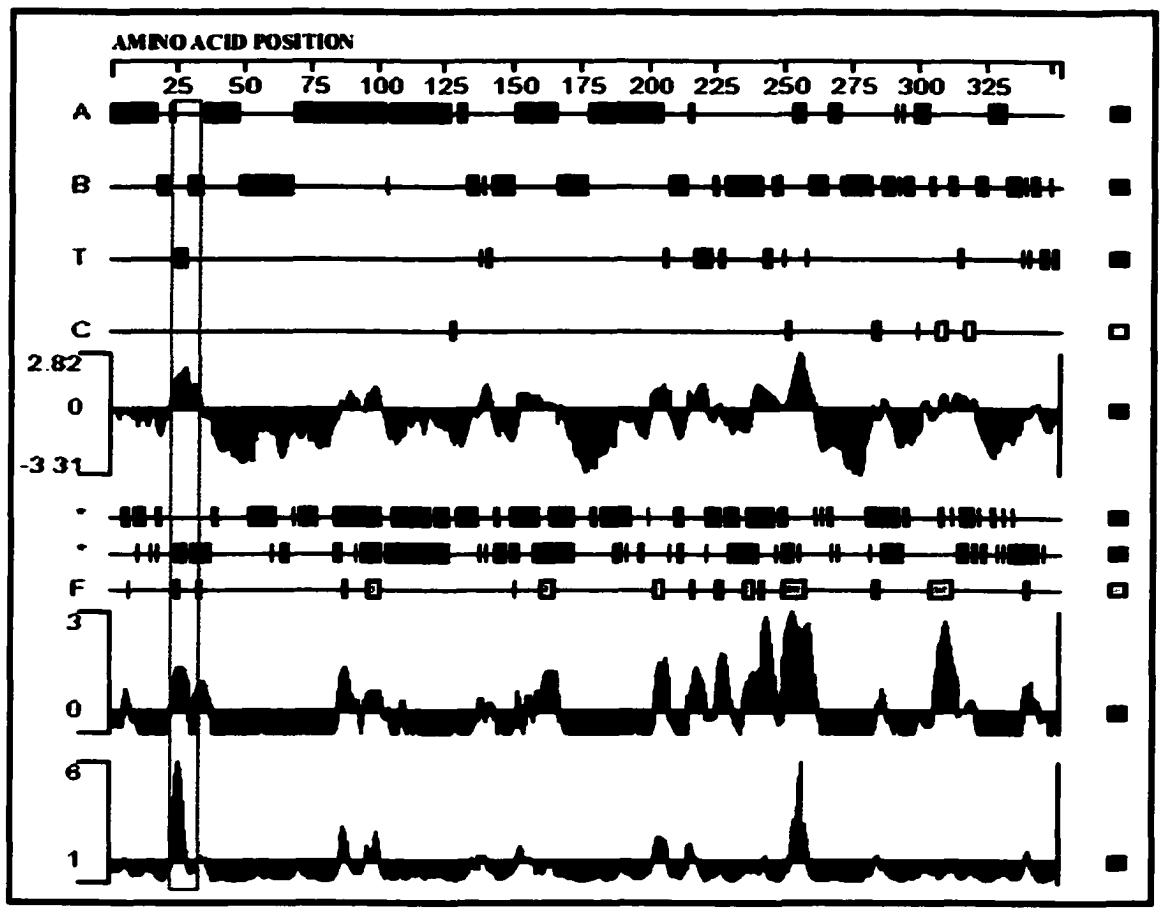


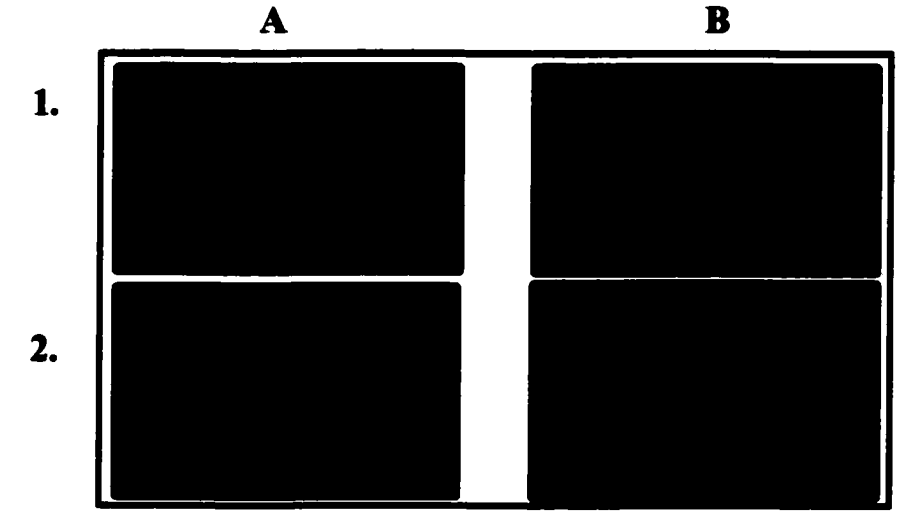
Figure 4.4. ***Analysis of the protein sequence predicted by the T. simiae ORF1 using Protean™, analysis software.***

The boxed area represents amino acids 25 to 33 which comprised the synthetic B cell epitope.

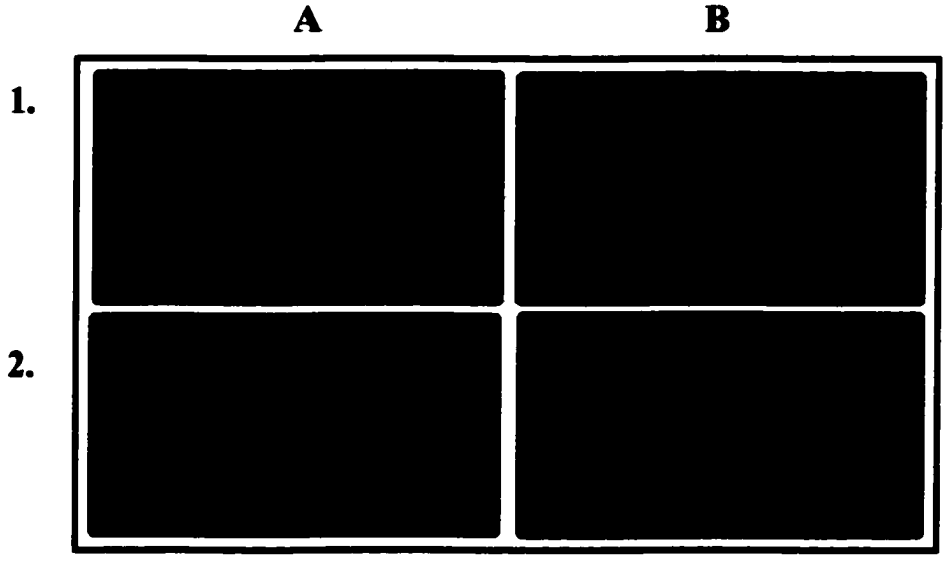
4.3. *Immunofluorescence analysis of PCF using the T. simiae peptide-specific mouse antiserum*

The mouse antiserum specific for the *T. simiae* ORF 1-B cell epitope was used for immunolocalisation of the protein by immunofluorescence using both live and fixed, permeabilised PCF of *T. simiae* and *T. congolense*. No immunofluorescence was observed on live PCF of *T. simiae* and *T. congolense* (upper panel, Figure 4.5) whereas bright fluorescence was observed on both species after acetone or paraformaldehyde fixation (lower panel, Figure 4.5). The fluorescence patterns suggest that the protein is localised to the plasma membrane and to what look like internal membranes in organelles, although resolution was not enough to state this with certainty. No fluorescence was observed on either live or fixed parasites with the preimmune mouse serum (Figure 4.5).

The results indicate that the TS ORF 1 may encode an internal, membrane - associated polypeptide that is expressed in procyclic forms of both *T. simiae* and *T. congolense*. It is also possible however, that the epitope represented by the synthetic peptide used for immunization is a cross reacting B-cell epitope shared by unrelated membrane proteins of *T. simiae* and *T. congolense*. The lack of surface fluorescence on live parasites suggests that even if the protein is surface disposed, the epitope is not. An alternative is that the molecules are internally orientated.



Immunofluorescence on live parasites



Immunofluorescence on fixed, permeabilised parasites

Figure 4.5. ***Immunofluorescence on PCF using a mouse antiserum against the T. simiae predicted B cell epitope.***

A: T. simiae. B: T. congolense. 1: Immune serum. 2: Pre-immune serum.

4.4. *Localisation of the T. simiae ORF1 B-cell epitope by immunogold electron microscopy.*

Although the immunofluorescence results on permeabilised parasites did provide visual evidence that the gene product may be a membrane-associated protein, this localization was equivocal. I therefore attempted immunogold localisation using the TS ORF 1 peptide - specific mouse antiserum as the primary antibody.

The ultrastructure of *T. simiae* PCF was initially examined by transmission electron microscopy, as shown in Figure 4.6. The micrographs show that *T. simiae* PCF has a surface coat as expected. The surface coats of the PCF show blebbing of surface proteins. These proteins may be procyclins expressed in *T. simiae* PCF, as similar micrographs were previously observed with *T. brucei*. The other distinctive feature is the presence of large internal vacuoles.

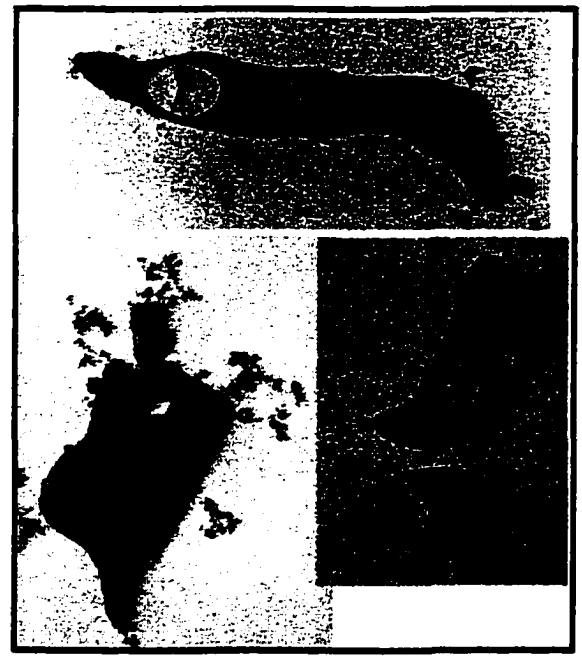


Figure 4.6. ***Ultrastructure of T. simiae PCF as visualized by transmission electron miscroscopy (20K magnification).***

Immunolocalisation of the TS ORF 1 B-cell epitope in the parasites was attempted by immunogold labelling. Ultrathin, Epon / Araldite - embedded sections of both *T. simiae* and *T. congolense* PCF were used and bound mouse immunoglobulins were detected by goat anti-mouse immunoglobulins conjugated to10 nm gold particles. Mouse pre-immune serum was used as a negative control. The results are shown in Figure 4.7.

Although the labelling was weak, Gold particles were located within vacuoles as well as on the membranes of both *T. simiae* and *T. congolense.* The localisation of the 10 nm gold particles in the vacuoles of the parasites was puzzling. Though we were able to see some localisation of the gold particles on the membranes of the parasites, most of the labelling seemed to occur in vacoules. I concluded that the immunolabelling was marginal, perhaps due to the plastic embedding and failure of the antibodies to penetrate the sections. Perhaps ultrathin cryosections would solve this problem as this method of fixation aids in preservation of epitopes and allows antibody penetration into cells and tissues. This technology was unfortunately not available at the University of Victoria.

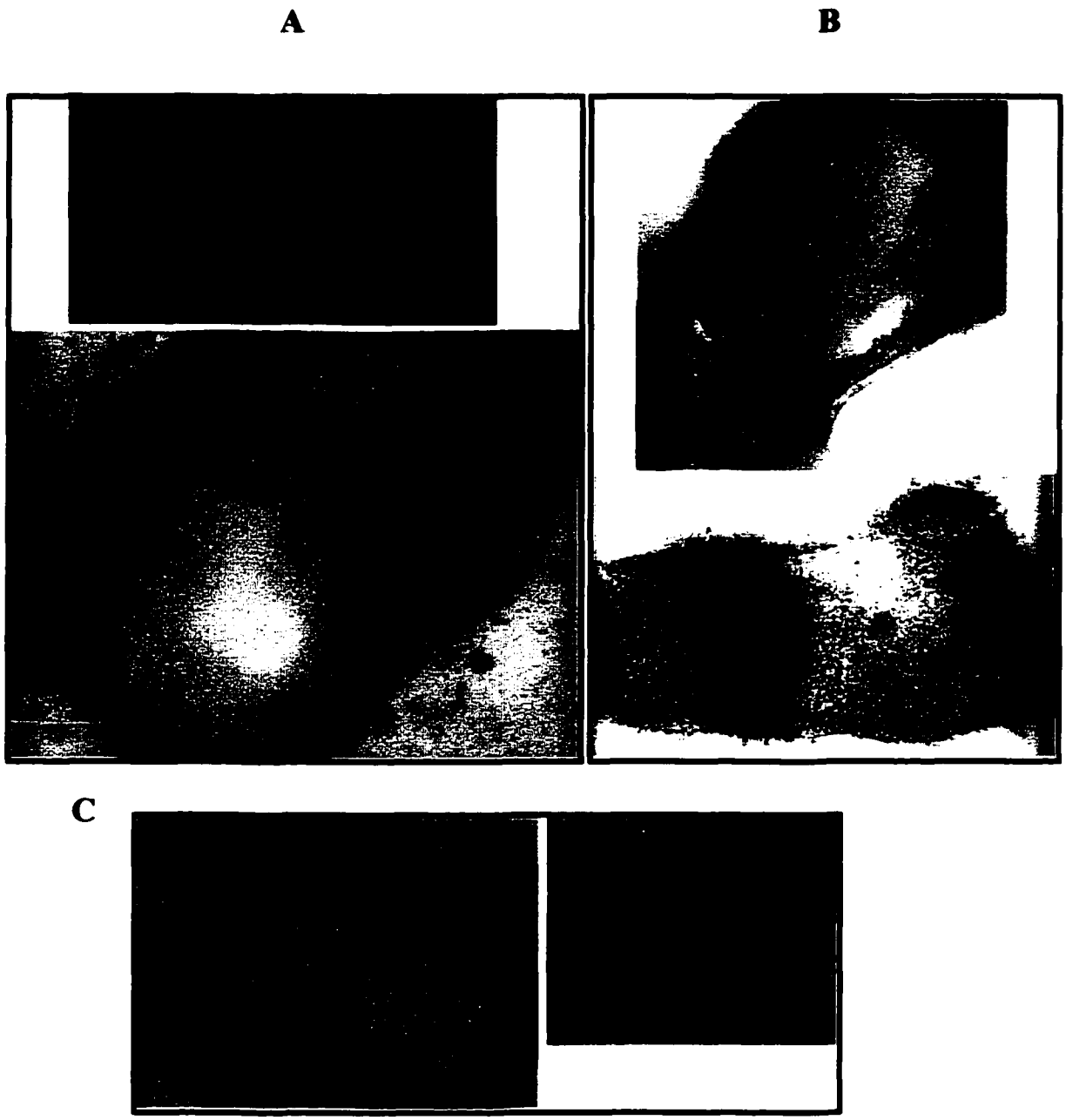


Figure 4.7. ***Immunogold labelling using the T. simiae ORF 1 B-cell epitope-specific mouse antiserum on ultrathin sections of T. simiae and T. congolense PCF.***

A: T. simiae with the peptide- specific antiserum. B: T. congolense with the peptide specific antiserum. C: T. simiae with pre-immune serum (negative control).

4.5. *Detection of the novel T. simiae protein by immunoblotting*

Immunoblots were performed on lysates of both *T. simiae* and *T. congolense* PCF using the *T. simiae* ORF 1 B-cell epitope-specific mouse antiserum. The results are shown in Figure 4.8. A band at approximately 30 kDa was seen with lysates from both *T. simiae* (panel A, lane 1) and *T. congolense* (panel A, lane 2). This broadly corresponds to the predicted mass of the protein (37 kDa). Lower bands below 21.5 kDa were also detected. Perhaps these are degradation products or different forms of the protein. When compared to immunoblots using surface directed *Nannomonas* – specific mAb TC 491 (panel C, lane 1; *T. simiae*. Lane 2; *T. congolense*), the immunoreactivity of the peptide-specific antiserum is well within the expected range of the antigenic, carbohydrate containing surface disposed molecules analysed from both *T. simiae* and *T. congolense* PCF. Pre-immune serum was used as a negative control, and as expected, no immunoreactivity was seen with either *T. simiae* (panel C, lane 1) or *T. congolense* lysates (panel C, lane 2).

Taken together, the immunofluorescence and immunoblotting results indicate that TS ORF 1 encode a membrane protein that is expressed in procyclic parasites of the subgenus *Nannomonas*.

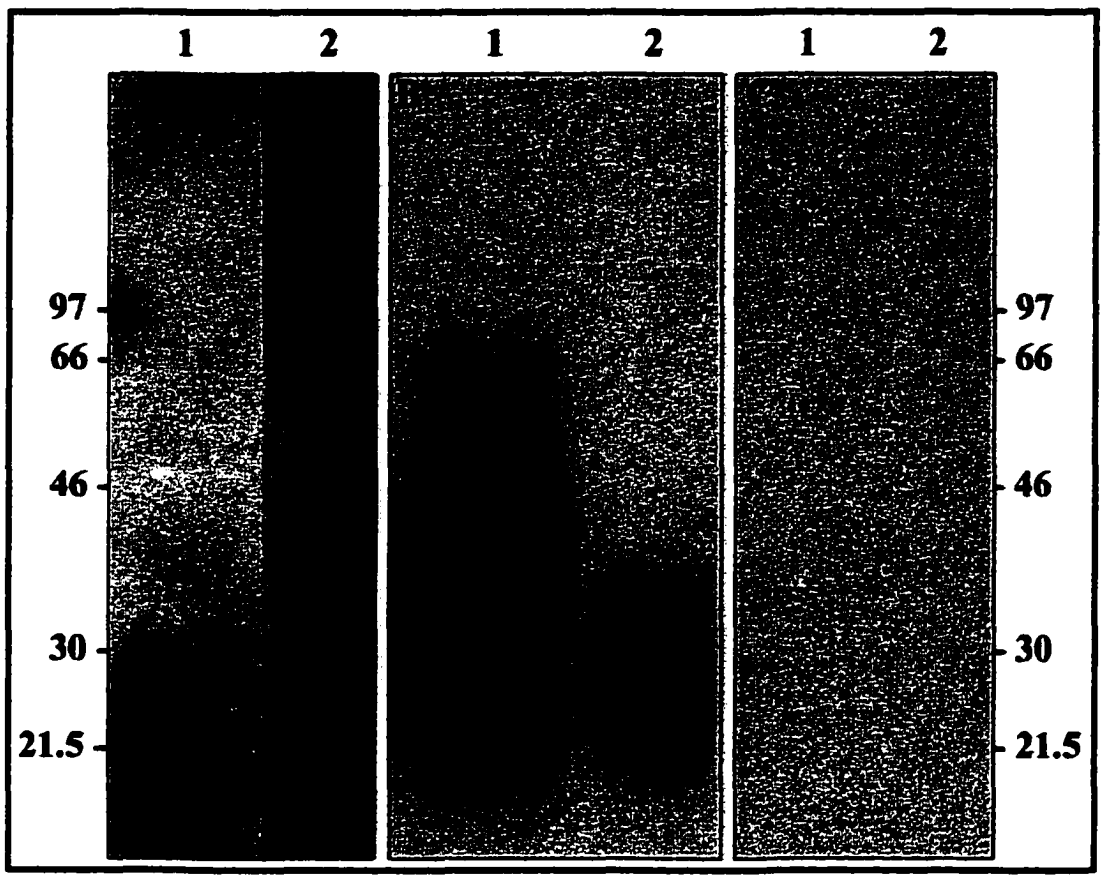


Figure 4.8. ***Immunoblot analysis of T. simiae and T. congolense PCF using anti-ORF 1 B-cell epitope antiserum.***

A: Mouse anti-KLH-conjugated peptide (RREHYTVGR) was used as the primary antibody.
B: MAb TC 491 (anti-T. congolense) was used as the primary antibody (positive control).
C: Pre-immune mouse serum was used as the primary antibody (negative control).

Lane 1: T. simiae CP11 lysate.
Lane 2: T. congolense K45/1 lysate.
Apparent molecular masses are shown in kiloDaltons on the right side of the figure.

4.6. *Derivation of monoclonal antibodies against deglycosylated HPLC-purified molecules from T. simiae PCF.*

In a final attempt to identify polypeptide moieties of *T. simiae* PCF, I derived monoclonal antibodies against deglycosylated membrane molecules. HPLC-purified molecules from *T. simiae* PCF were chemically deglycosylated using anhydrous TFMSA and used as the immunizing antigen. More than 100 hybridoma clones were obtained. Seven were selected by ELISA (using the deglycosylated molecules as solid- phase antigen), but none of these bound to the surface of *T. simiae* or *T. congolense* PCF, as tested by both immunofluorescence on live parasites and flow cytometry (results not shown). These mAbs were then tested on total lysates of *T. simiae* and *T. congolense* PCF by immunoblotting and four showed strong reactivity. These were also tested by immunoblotting on both glycosylated and deglycosylated HPLC-purified molecules from *T. simiae* PCF.

Immunoblot profiles on total lysates of *T. simiae* PCF using three selected, representative mAbs are shown in Figure 4.9. A broad smear from 30 kDa to 70 kDa was observed with the mouse immune serum (lane 1; positive control). Preimmune serum showed no binding (not shown). A very dark broad band centered at approximately 66 kDa was seen with mAb 1E1 (lane 2). In addition, this mAb 1E1, mAb 2D8 and mAb 2F6 showed a dark smear between 32 and 48 kDa, in some cases showing a doublet. Curiously, no immunoreactivity was seen with mAbs 2D8 and 2F6 as primary antibodies on lysates on *T. congolense* PCF (not shown), indicating that they recognise a *T. simiae*-specific epitope. The same pattern was observed using mAb 1G1 (Figure 4.10, panel A),

although this mAb cross-reacted with the lower band in *T. congolense* PCF. None of these bands were detected in lysates of *T. brucei* (lane 2). This indicated that mAb 1G1 recognises a cross-reacting epitope in *T. simiae* and *T. congolense* PCF and thus appears to be specific for the subgenus *Nannomonas*. In contrast, mAb 1E1, which reacted with the 66 kDa band of *T. simiae* (lane 2, Figure 4.9), reacted with molecules from procyclic trypanosomes of all species tested (Figure 4.10, panel B). It is interesting that a dark band was observed also at 150 kDa in this somewhat overexposed blot. Cross reactivity with other kinetoplastid parasites was not tested.

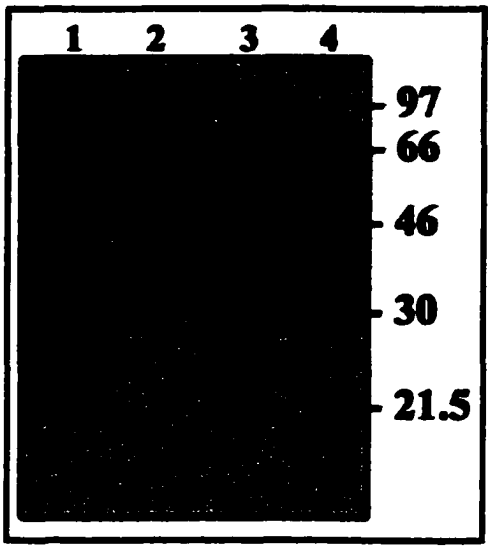


Figure 4.9. ***Immunoblot on total lysates of T. simiae PCF using mAbs raised against deglycosylated, HPLC-purified molecules from T. simiae PCF.***

Lane 1: mouse immune serum (positive control). This antiserum was from the mice that were subsequently used for spleen cell donors for hybridoma production.
Lane 2: mAb 1E1. Lane 3: mAb 2D8. Lane 4: mAb 2F6.
Apparent molecular masses are shown in kiloDaltons on the right hand side of the figure.

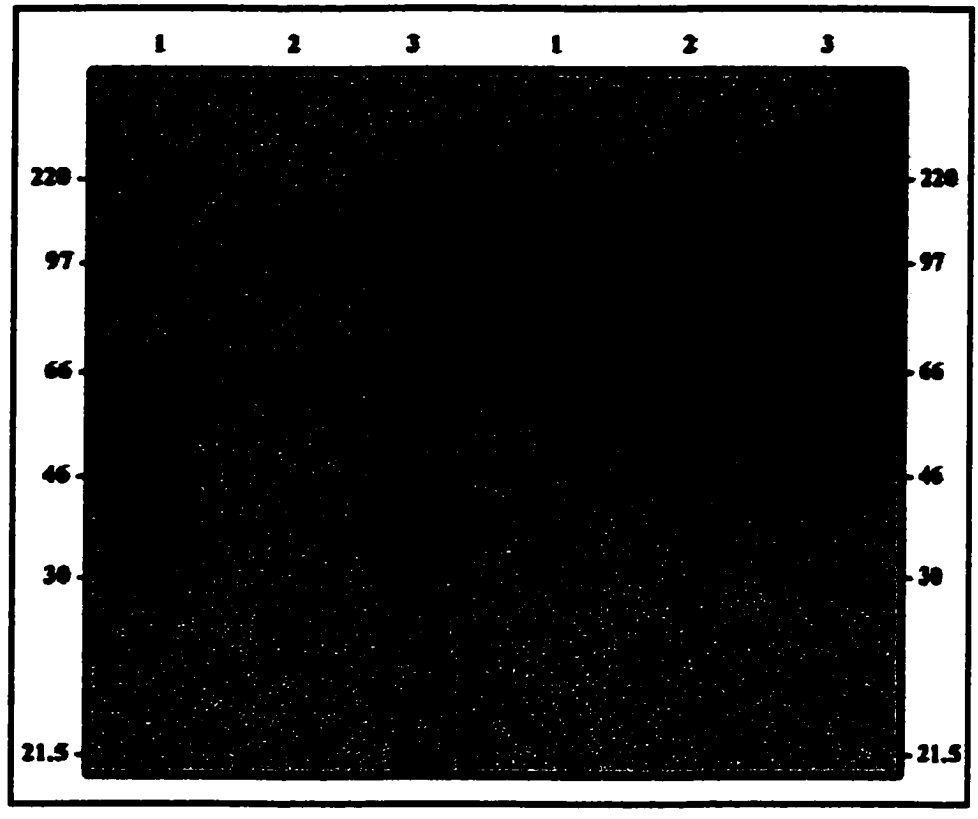


Figure 4.10. ***Immunoblot analysis of lysates of procyclic trypanosomes using selected mAbs.***

Panel A: mAb 1G1. Panel B: mAb 1E1.
Lane 1: T. simiae CP11. Lane 2: T. brucei 427/1. Lane 3: T. congolense K45/1.
Apparent molecular masses are shown in kiloDaltons.

Immunoblots were also performed on HPLC-purified molecules from *T. simiae* PCF both before and after deglycosylation (Figure 4.11). It was puzzling that mAb 1E1 showed no immunoreactivity with the HPLC fractions or the deglycosylated, HPLC-purified molecules from *T. simiae* PCF (not shown). This might be due to the fact that the proteins recognised by this mAb are not present in significant quantities in the HPLC-purified material and thus the mAb only reacts with antigen in total lysates of the

parasites. In contrast, mAb 1G1 showed strong reactivity with the HPLC-purified molecules, both glycosylated and TFMSA - treated (Figure 4.11). This mAb is interesting as it may recognise a polypeptide that is *Nannomonas* specific and may prove useful for immunoaffinity purification of the antigen and allow its identification by mass spectrometry.

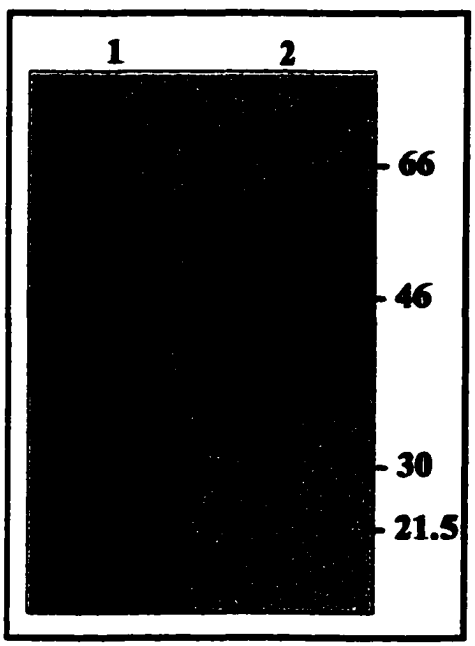


Figure 4.11. ***Immunoblot analysis of glycosylated and deglycosylated HPLC-purified molecules of T. simiae PCF using mAb 1G1.***

MAb 1G1 was used as the primary antibody.
Lane 1: Deglycosylated, HPLC-purified molecules. Lane 2: HPLC-purified molecules.
Apparent molecular masses are shown in kiloDaltons.

Finally, as seen in Figure 4.12, immunoblots with both mAb 2D8 (panel A) and mAb 2F6 (panel B) showed a broad smear from 35 kDa to 60 kDa with the HPLC-purified fraction of *T. simiae* PCF (lane 2), which clearly indicates that the immunoreactive molecules may be glycosylated. However, no band was seen with either

of these mAbs as the primary antibody in immunoblots with the deglycosylated immunizing antigen. This again reflects the fact that the deglycosylated molecule is not present in significant amounts to be detected by immunoblots or is of low molecular mass and not resolved on the 10 % SDS-PAGE gels and thus is not detected on the immunoblots.

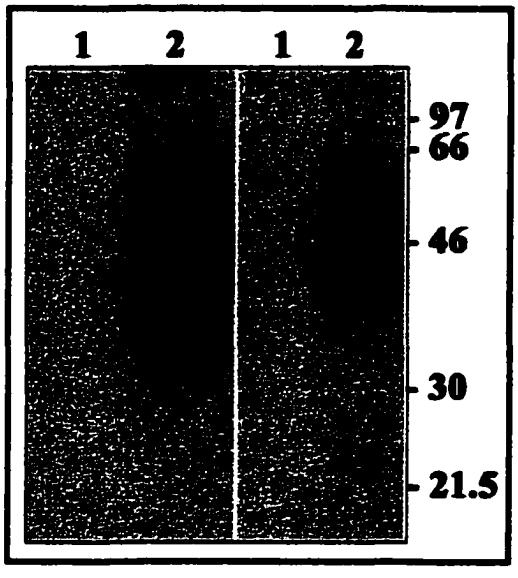


Figure 4.12. ***Immunoblot analysis of glycosylated and deglycosylated HPLC-purified molecules of T. simiae PCF using mAb 2D8 and mAb 2F6 as the primary antibody***

Panel A: mAb 2D8. Panel B: mAb 2F6.
Lane 1: Deglycosylated, HPLC-purified molecules from T. simiaePCF.
Lane 2: HPLC-purified molecules from T. simiae PCF.
Apparent molecular masses are shown in kiloDaltons.

Although we did not further characterise any of the proteins recognised by these selected mAbs, it is evident that all of the immunoreactivity is with molecules in the range of 35 kDa to 60 kDa, which suggests that they probably recognise epitopes within

molecules that are procyclin analogues of *T. simiae*. Hence, these mAbs will be useful in the future as valuable tools for identification of such molecules.

Summary

Attempts to obtain primary protein sequences for polypeptides comprising the cell surface glycoconjugates of *T. simiae* PCF proved extremely difficult. In more than 2 years work, only one amino acid sequence was obtained from an internal peptide of CNBr-digested molecules in a mixture of species enriched by reverse-phase HPLC purification. In contrast, sequences of several peptides from *T. congolense* were relatively easily obtained by gas-phase protein microsequencing. Sequences from both *T. congolense* and *T. simiae* were also obtained by Q-TOF mass spectrometry. Only one protein was identified by mass spectrometry however and this was GARP from *T. congolense*. It is possible that the *T. simiae* polypeptide moieties were in low abundance compared to those of *T. congolense*. The *T. simiae* internal sequence was used to design PCR primers that allowed amplification of cDNA sequences and determination of an open reading frame (TS ORF 1). This entire ORF sequence was used to search the NCBI non-redundant database and no matches were found. An antiserum was raised to a predicted B-cell peptide epitope in this ORF and used in immunofluorescence and immunoblotting assays to localise the epitope to membrane - associated regions of *T. simiae* and *T. congolense* PCF and to a polypeptide in both species that was approximately the expected mass. Both immunofluorescence and immunoblotting reactions on *T. brucei* PCF were negative, indicating that the polypeptide was specific to the subgenus *Nannomonas*. Although the protein appeared to localise to

the plasma membrane and to membrane regions inside the parasite by immunofluorescence and by immunogold electron microscopy, this assignment was not definitive. The 1056 bp ORF DNA sequence was submitted to the Genbank database. Interestingly, this was only the third sequence for *T. simiae* in the database, the others being sequences of a mini-exon and a ribosomal protein. Monoclonal antibodies were raised against the HPLC-enriched, deglycosylated *T. simiae* molecules in order to obtain polypeptide-specific reagents. Several mAbs were obtained and showed reactivity on lysates of parasites in immunoblotting experiments. The mAbs were shown either to be subgenus *Nannomonas* specific or to react with parasites of both subgenus *Trypanozoon* and *Nannomonas*. The complexity of the immunoblotting patterns obtained and the difficulty encountered in assigning the mAb specificity to defined molecules reinforce earlier observations with *T. brucei* spp. and *T. congolense* that there are multiple glycoconjugates on the surface of PCF. The identification and primary structure analysis of several of these in *T. brucei* was accomplished by novel mass spectrometry approaches and was possible because the procyclin loci had been completely sequenced in the trypanosome clone used [Acosta-Serrano *et al.*, 2001]. Primary structures of GPI-anchored glycoconjugates in *T. congolense* were not obtained [Bütiköfer *et al.*, in press] because of difficulties similar to those encountered with *T. simiae* PCF during the research for this thesis.

5. Concluding Comments and Future Directions

Identification and characterisation of the procyclins expressed on the surface of *T. brucei* spp. procyclic forms has taken more than 10 years and has involved several laboratories in Europe and North America. These molecules are all glycoproteins and although the two major forms were identified after much effort, they were all amenable to primary structural analysis by protein and DNA sequencing. This has not been the case with *T. congolense* and *T. simiae* procyclic forms. Only one polypeptide or gene sequence has been determined in parasites of this subgenus (*Nannomonas*), the glycine-alanine-rich protein (GARP) of *T. congolense*. Research on the cell surface molecules of *T. brucei* spp. [reviwed in Roditi *et al.*, 1998], *T. congolense* [Stebeck and Pearson, 1994; Bütikofer *et al.*, in press] and *T. simiae* [this thesis] has revealed that all of them are heavily glycosylated and contain GPI-anchors. With *T. brucei* spp., the most well studied parasites, the procyclins have been shown to present a dynamic mosaic of surface molecules that changes during differentiation of the trypanosomes in the tsetse fly and are thought to play a central role in this process [Pearson, 2001]. Although it has been clearly demonstrated that the EP form of procyclin is required for establishment of heavy midgut infections in tsetse [Ruepp *et al.*, 1997] and that GARP can restore midgut establishment of EP- negative mutants [Ruepp *et al.*, 1999], there are little data supporting other roles for procyclins. Thus it was the major goal of my thesis to compare the surface molecules of two species of trypanosomes that have the same life cycle in the vector. These parasites should have similar functional moieties on their surfaces if they interact with molecules of the tsetse.

Data presented in this thesis showed that:

- Major, preponderant surface glycoconjugates are expressed on procyclic culture forms of both *T. simiae* and *T. congolense.*

- The major surface glycoconjugates are immunodominant since all surface directed monoclonal antibodies recognise them.

- The epitopes on the surface glycoconjugates that are recognised by the monoclonal antibodies are specific to the subgenus *Nannomonas.*

- The two major surface glycoconjugates from both *T. simiae* and *T. congolense* are broadly biochemically similar although they do have slightly different elution profiles from HPLC-reverse phase columns, different migration patterns on SDS-PAGE gels and different staining characteristics. Thus they are biochemically different.

- The two species of parasites have at least one major difference in their expressed surface molecules since:

a) The GARP gene is not present in *T. simiae* clone CP11 as shown by Southern blotting under conditions of low-moderate stringency.

b) The GARP protein is not expressed in *T. simiae* CP11 as shown by immunoblotting with an anti-GARP polypeptide rabbit antiserum and by Q-TOF mass spectrometry.

- Sequencing of peptides by Q-TOF mass spectrometry showed that *T. simiae* surface molecules have some unique polypeptide sequences that are not found in the NCBI non-redundant database, i.e. they are unique to *T. simiae* procyclic forms.

- The GPI anchors of the surface molecules show differences between *T. simiae* and *T. congolense* as revealed by differential binding of proaerolysin toxin.

- The presence of cross-reactive carbohydrate epitopes on different polypeptides (eg. GARP) indicates that *T. simiae* and *T. congolense* procyclic trypanosomes place the same carbohydrate "coats" on different "hangers". This phenomenon suggests that the carbohydrate moieties are the specific ligands for molecular interactions between trypanosomes and tsetse.

- A search for polypeptide sequences of *T. simiae* that may be the equivalent of GARP in *T. congolense* was not successful, although the peptide sequences of *T. simiae* identified by Q-TOF mass spectrometry may prove to be candidates worth pursuing.

If it is true that carbohydrates exposed on the surface of trypanosomes of the subgenus *Nannomonas* are functionally important in the parasite life cycle then future research will require carbohydrate and protein chemical approaches of high resolution and sensitivity. Clearly, a comparative analysis of different species of African trypanosomes during transit through the tsetse vector will be valuable. There is no doubt that characterisation of molecules on procyclic surfaces will give clues as to structure-

function relationships. Perhaps direct approaches to function will be attainable by measuring the effects on the parasite life cycle by feeding tsetse flies purified carbohydrate portions of trypanosome surface glycoconjugates or by interference using selected monoclonal antibodies. Only by obtaining precise molecular information will rational strategies be achieved for interference in trypanosome transmission by the bane of Africa, the tsetse.

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