Antigen Variation:
Untersuchungen zur GPI-Verankerung der varianten Oberflächenglycoproteine in *Trypanosoma brucei*

Antigenic Variation:
Investigation of GPI-Anchoring of the Variant Surface Glycoprotein in *Trypanosoma brucei*

DISSERTATION

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List of Abbreviations

AMC	7-amino-4-methylcoumarin
BSA	Bovine serum albumin
CBD	Chitin binding domain
CGA	Citrat glucose anticoagulante
CRD	Cross-reacting determinant
kDa	Kilo Dalton
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxid
DTT	1,4-Dithiothreitol
EDTA	Ethylendiamin-tetraacetat-dinatriumsalzdihydrate
EGTA	Ethylenglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetate
ER	Endoplasmic reticulum
eIF	Eukaryonitic initiation factor
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GPI-PLC	GPI-specific phospholipase C
HBS	HEPES buffered saline
HDZ	Hydrazine
HEPES	N-(2-hydroxyethyl)piperazin-N'-(2-ethansulfonic acid)
HRP	Horseradish peroxidase
IC_{50}	Concentration, which cause 50% inhibition
IgY	Gamma immunoglobin from egg-yolk
IPTG	Isopropylthiogalactosid
kb	Kilo base
MBP	Maltose binding protein
MITat	Molteno institute trypanozoon antigenic type

NP-40	Nonidet P-40
PBS	Phosphate buffered saline
pCMPSA	p-chloromercuriphenylsulfonic acid
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMS	Post mitochondrial supernatant
PMSF	Phenylmethylsulfonylfluorid
RNase	Ribonuclease
SDS-PAGE	Polyacrylamid gel electrophoresis with sodium dodecyl sulfate
TBS	Tris buffered saline
TCA	Trichloro acetic acid
TEMED	N,N,N',N'-tetramethylethylendiamine
Tris	Tris(hydroxymethyl)aminomethane
VSG	Variant surface glycoprotein
mfVSG	Membrane form VSG
sVSG	Soluble VSG

1 Introduction

1.1 Trypanosomes

1.1.1 *Trypanosoma brucei* as Pathogen of Sleeping Sickness and Nagana

Trypanosomes are monocellular, parasitic eukaryotes. Since the beginning of last century, they have been well known as pathogens of some tropical diseases of human and animals. Sleeping sickness of human is caused by *Trypanosoma brucei gambiense* (Dutton, 1902) and *Trypanosoma brucei rhodesiense* (Stephens & Fantham, 1910). These two subspecies are similar in morphology, however, they differ in the type of diseases been caused. *T. brucei rhodesiense* causes the acute form of trypanosomiasis in East Africa, whereas *T. brucei gambiense* induces the chronic form of trypanosomiasis in West Africa. The acute sleeping sickness leads to death after a few months and the chronic form results to death within 3 years if the illness is not been treated. According to the report of World Health Organization (WHO), about 55 million Africans live in endemic regions, and about 500,000 people are newly infected every year.

The third subspecies, known as *Trypanosoma brucei brucei*, is not pathogenic for human, because this parasite is lysed by a component of the human serum (haptoglobin of HDL) (Hajduk & Hager, 1994). It infects domestic animals (cattle, sheep, dogs, pigs and goats) and many other wild animals. The trypanosomiasis caused in domestic animals has important economic significance (Nagana).

All these trypanosomiases are transmitted by the tsetse fly, which injects the infective metacyclic forms into the blood during feeding. The sleeping sickness and Nagana-plague are restricted to tropical Africa in a breadth of 15 degree North and 18 degree South (Tsetse-belt) (Dönges, 1988).

There are available chemical therapies (suramine or berenil, respectively) against sleeping sickness and Nagana-plague in the early stage of infection. Treatment becomes very difficult, however, in the later stages of the infection because of side effects and the limitation of α-difluoromethylornithine (DFMO) in treatment of *T. b. gambiense* infection (Doua & Boa-Yapo, 1994).

Study of the metabolism of *T. brucei* has therefore significance for the development of new anti-trypanosome medicine. Although the pathogenic species have received most attention for the disease they cause, these organisms have also provided good models for the study of various fundamental biological phenomena because of their unusual features.

1.1.2 Classification

Trypanosome belongs to the family *Trypanosomatidae*. The genus *Trypanosoma* consists of blood or tissue parasites of vertebrates with invertebrate intermediate hosts. Hoare (1966) divided the genus *Trypanosoma* into two sections: A. *stercoraria*, B. *salivaria*, on the basis of whether they develop in the hindgut or in salivary glands of the insect vector. The species *T. brucei* belongs to the *salivaria* section. The following classification is based on Levine *et al.* (1980):

Phylum	Protozoa	
Subphylum	Sarcomastigophora	
Class	Mastigophora	
Subclass	Zoomastigophora	
Order	Kinetoplastida	
Family	Trypanosomatidae	
Genus	Trypanosoma	
Species	Trypanosoma brucei	
Subspecies	Trypanosoma brucei brucei	(non human pathogenic)
	Trypanosoma brucei gambiense	(human pathogenic)
	Trypanosoma brucei rhodesiense	(human pathogenic)

1.1.3 Life Cycle

Transmission from one vertebrate host to another is carried out by blood-sucking invertebrates, usually an insect. Shortly after infected blood enters insect gut, bloodstream forms become transformed into insect gut forms; the latter give rise to the epimastigote forms and finally metacyclic forms. Upon an insect bite, the tsetse fly deposit metacyclic trypanosomes in the dermal connective tissue, inducing a local inflammatory reaction. From this region the parasite migrates to the lymphatic and hence to the bloodstream. From these sites, after undergoing intense multiplication, they penetrate the blood and

lymph capillaries, into the connective tissue and, after a month or so, cross the choroids plexus into the brain and cerebrospinal fluid. In the early stages of infection 'slender' forms predominate, eventually they give way to 'stumpy' forms via an 'intermediate' form stage. The invasion of the nervous system results in 'sleeping sickness', characterised by a general physical and mental depression and a desire to sleep. *T. b. gambiense* produces a chronic human disease that may last for several years before ending in death. In contrast, the disease caused by *T. b. rhodesiense* is an acute disease, which lasts for about six months before terminating fatally. The only difference between these species and *T. b. brucei* appears to be their ability to infect man (Smyth, 1994)

1.1.4 Morphology

A typical 'trypanosome' is an elongated organism, 15-30 μm in length (including flagellum) and 1.5-3 μm in width, with a single nucleus containing a large central nucleolus (karyosome). Movement is effected by a single flagellum, which originates in the *basal body*. In addition to a plasma membrane, an outside coat covering this membrane consists of about 10^7 virtually identical copies of a single glycoprotein known as variant surface glycoprotein (VSG). There are up to several hundreds genetically encoded variants of VSGs, which can be sequentially expressed. VSG apparently protects the parasite from the host's immune attack. In all trypanosomes this glycoprotein coat is lost after entering the vector. The endoplasmic reticulum is represented by tubules, which emerge from the nuclear envelope and ramify though the cytoplasm. The Golgi apparatus is generally located anteriorly opposed to the cytopharynx. It has the usual eukaryote structure with stacks of cisternae. The kinetoplast is a filamentous body, which contains kinetoplast DNA and has been shown to be part of the mitochondrion. Unlike mitochondria DNA of other cells, the synthesis of kinetoplast DNA is temporarily linked to nuclear DNA synthesis. Ultra structure studies have revealed marked cytological and metabolic differences between the bloodstream and fly gut forms (Vickerman, 1962, 1985). In the slender bloodstream forms, the mitochondrion forms one single tube extending the length of the body, with few cristae present. In contrast, in the fly gut form, the mitochondrion takes the form of an extensive tubular network with well- developed cristae.

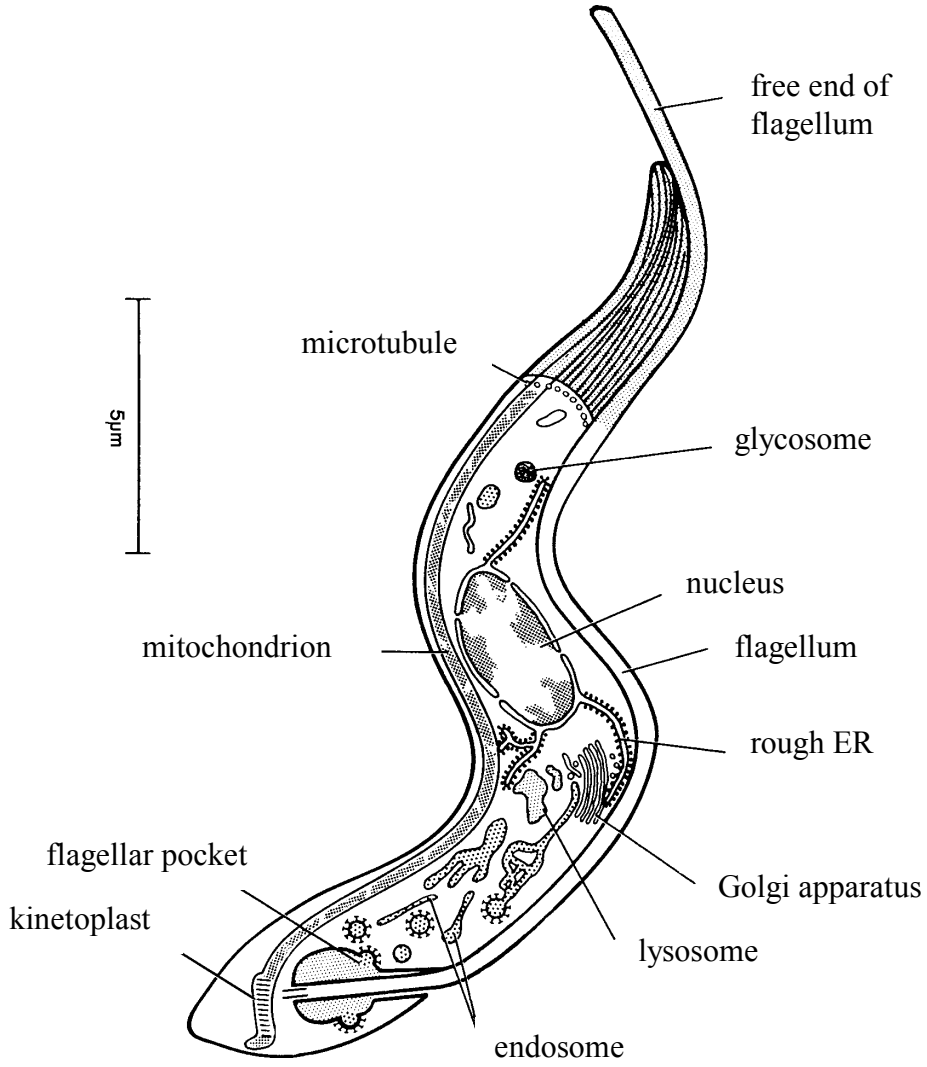

Fig. 1.1: Cell Structure of the Long Slender Blood form of *Trypanosoma brucei* (From Ilrad, 1988)

1.1.5 Unusual Features

Trypanosomes have excited the interest and attention of molecular biologists, immunologists and geneticists on account of a number of unusual features. Probably due to the early divergence of this eukaryotic lineage in evolution, studies of the molecular biology of this cells have produced a plethora of novel and significant discoveries, which include polycistronic transcription, trans-splicing of precursor mRNAs, the presence of an unique organelle containing

most of the glycolytic enzymes, editing of mitochondrial mRNAs by uridine insertion/deletion, antigenic variation and glycosylphosphatidylinositol anchors of membrane proteins. All of these make them interesting models for the study of various fundamental biological phenomena.

1.2 VSG-Surface Coat

1.2.1 Antigenic Variation

The surface coat of the bloodstream form of African trypanosomes is arranged as a dense monolayer of homodimers on the parasite surface. The ability of the surface coat of trypanosomes to undergo continuous antigenetic variation results in the escape from the host's immune response. The surface antigens change every 8 ~ 10 days after the trypanosome entered the vertebrate host. Specific antibodies produced by the host are no longer effective owing to the appearance of new variant surface antigens (VSGs). The exchange of VSG is based on the sequential expression of several hundreds different VSG genes (Van der Ploeg, 1982). The actively transcribed gene is always located in a *VSG expression site,* which is in a polycistronic transcription unit on the telomer of a chromosome. There are probably 20~40 *expression sites* within the genome*,* but only one of them is active at a given time. The *switching* to another VSG is effected by two different ways. One is by *in situ* activation of a new *expression site*; the other is by the rearrangement of the DNA on the active *expression site.*

1.2.2 Molecular Structure of the VSG

The VSG is attached to the plasma membrane by means of a glycosylphosphatidylinositol (GPI) anchor. VSG consists of 2 domains, one N-terminal domain with 350~400 amino acids and one C-terminal domain with 50~100 amino acids (Carrington & Boothroyd, 1996) and has at least one N-linked oligosaccharide, although the position of this oligosaccharide varies (Cross, 1984; Turner & Barry , 1989). The carbohydrate content varies from 7 to 17% in a range of glycoproteins studied (Johnson & Cross, 1977). The sequence of the N-terminal domain is extremely diverse except for the relative location of cysteine residues, which are conserved (Cross, 1984). The C-terminal domain

exhibits some degree of sequence conservation (Holder & Cross, 1981). VSGs are found as dimers in solution (Auffret & Turner, 1981) and are believed to exist as dimers or higher oligomers on the cell surface (Strickler & Patton, 1982).

1.2.3 Biosynthesis of VSG

Precursors of GPI anchored proteins have a classical signal sequence at their N-terminus and a GPI anchoring signal at their C-terminus. The N-terminal signal directs the nascent polypeptide through the rough endoplasmic reticulum membrane and is removed as a co-translational event (McConnell *et al*., 1981). N-glycosylation appears to occur cotranslationally, but in a few cases N-glycosylation was delayed or incomplete (Ferguson *et al*., 1986). The C-terminal signal is necessary and sufficient to direct GPI addition (Caras *et al*., 1987; Moran & Caras, 1991; Moran *et al*., 1991; Kodukula *et al*., 1993). After completion of the polypeptide, the C-terminal GPI anchoring signal is removed and replaced by a preformed GPI anchor (Ferguson & Duszenko, 1986) (Fig. 1.2).

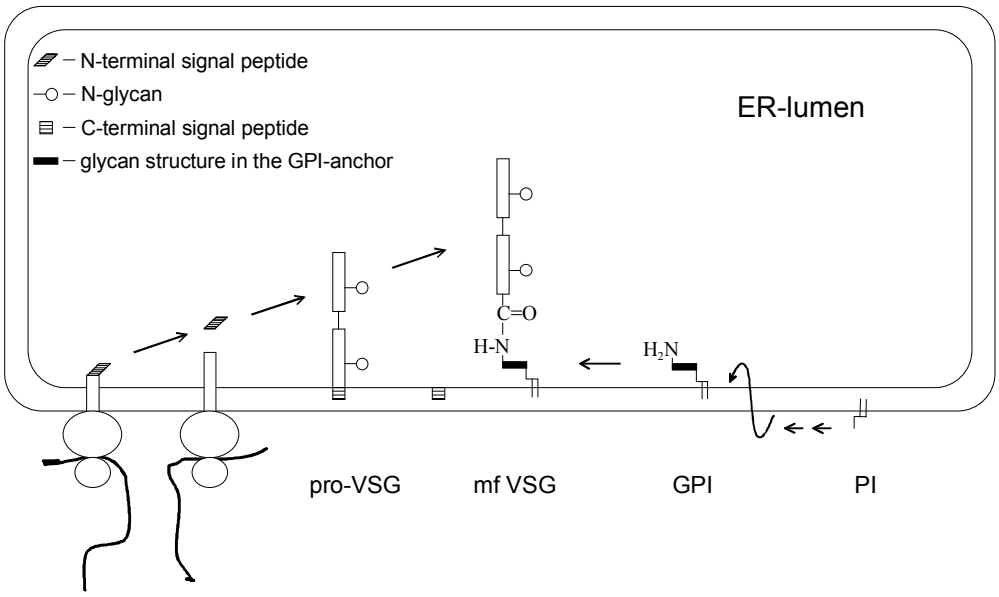

Fig. 1.2: Biosynthesis of the VSG Molecule (from McConville & Ferguson, 1993)

1.2.4 N-Glycosylation of VSG

VSGs may be grouped into 3 sub-types based on C-terminal sequence homologies (Carrington *et al*, 1991). Each VSG variant contains one, two or three N-linked oligosacchrides (Zamze, 1990; 1991). Type I VSGs contain one conserved N-glycosylation site about 50 residues from the mature C-terminus and contains oligomannose structures. Type II VSGs generally contain two glycosylation sites, one 5 or 6 residues from the mature C-terminus and the other about 170 residues from the mature C-terminus. The first site contains a mixture of oligomannose structures and larger polylactosamine-containing structures. The latter site is occupied by small structures like $Man_{3-4}GlcNAc_2$ and $GlcNAcMan_3GlcNAc_2$ or regular biantennary complex glycans. The one characterized type III VSG (MITat 1.5) contains three potential N-glycosylation sites (123, 356 and 377 residues from the mature C-terminus) consisting of a mixture of oligomannose and complex biantennary structures. The larger polylactosamine-containing structures and regular biantennary complex glycans contain galactose residues and linked to GlcNAc in a β1,4 glycosidic linkage. The observed differences in GPI anchor galactosylation (see 1.3.1) and N-glycan number and structure appear to correlate with VSG sub-classes. Therefore, it seems that the carbohydrate is used as space-filling material to compensate for differences in the VSG three-dimensional structures and to ensure the barrier characteristics of different VSG coats.

1.2.5 Transport of VSG to the Cell Surface, VSG-Recycling and Shedding

Intracellular transport of VSG to the cell surface occurs with a half time of about 15 min, where it is integrated into the surface coat by budding and fusion of small vesicles. VSG transport follows the classical secretory pathway: ER, Golgi apparatus, and delivery to the flagella pocket, where it is integrated into the surface coat. Incubation of trypanosomes at 20°C, a treatment that arrests intracellular transport from the trans-Golgi region to the cell surface in mammalian cells, caused the accumulation of VSG molecules in structures of the trans-Golgi network, and retarded the incorporation of newly synthesized VSG into the surface coat (Duszenko *et al*., 1988). VSG uptake by endocytosis was found to occur exclusively along the flagella pocket membrane and was related to the formation of coated pits and coated vesicles in this functionally

discrete surface domain. More than 95% of the endocytosed surface VSG is recycled back to the plasma membrane. Turnover and removal of old VSG are performed by VSG shedding at a rate of 2.2%/hr. After 7 days 87% and after 8 days 99% of the switched cell population has released all old VSG and is protected by a new and antigenically distinct surface coat (Duszenko & Seyfang, 1993).

1.3 GPI Anchor

1.3.1 Structure of the GPI Anchor

The GPI anchoring pathway seems to have been elaborated early in evolution since the core carbohydrate structure linking the protein to the lipid moiety is identical in all GPI anchors, namely protein-CO-NH-$(CH_2)_2$-PO_4-6Manα1-2Manα1-4GlcNH_2-myo-inositol-PO_4-lipid (McConville & Ferguson, 1993). This backbone is common to all GPI-anchored proteins found in eukaryotes, but is variously modified by side structures in different organisms and cell types (McConville & Ferguson, 1993). The structure of the inositol phospholipid also varies in different organisms. *Trypanosoma brucei* exclusively uses dimyristyl phosphatidyl-inositol at the blood stage and lysophosphatidylinositol at the insect stage. Cleavage of a GPI membrane anchor with bacterial PI-PLC (or eukaryotic GPI-PLC) leads to the formation of the inositol 1,2-cyclic monophosphate, which appears to be the major epitope involved in the cross reactivity of GPI anchored proteins (Zamze *et al*., 1988) (Fig. 1.3). The mannose residue next to glucosamine may be galactosylated. These α-Gal side-chains of the VSG GPI anchor are unusual structures, which appear to be restricted to African trypanosomes (Ferguson *et al*., 1988). Type I VSGs (MITat 1.4, MITat 1.6) contain 4 galactose residues per GPI molecule, type II VSGs (MITat 1.2) contain 8 galactose residues and type III VSGs (MITat 1.5) contain no galactose residue (Holder, 1985). The α-galactosylation machinery of African trypanosomes may be involved specifically to optimize the packing of different VSG coats, and therefore may increase the viability in the presence of host serum complement.

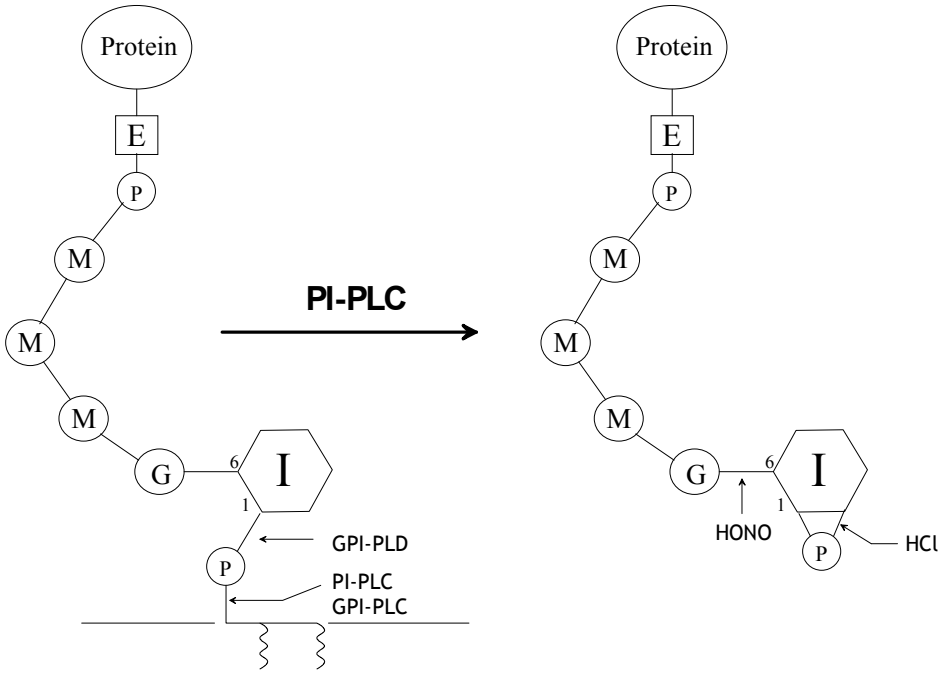

Fig. 1.3: Schematic Representation of the Structure of GPI Anchor and the Cross-reacting Determinant (CRD)

E: ethanolamine I: inositol P: phosphate
G: glucosamine M: mannose

1.3.2 Biosynthesis of the GPI Anchor

The relative abundance of the VSG protein in *Trypanosoma brucei* has made this organism extremely useful for the study of GPI anchor biosynthesis. The structure of the VSG GPI anchor is known (Ferguson *et al*., 1988) and principal features of the GPI biosynthetic pathway in trypanosomes were elucidated using a cell-free system based on washed trypanosome membranes (Masterson *et al*., 1989, 1990; Menon 1990). The GPI anchor precursors are synthesized in the outside of endoplasmic reticulum membranes by sequential addition of sugar and other components to phosphatidylinositol (Ferguson, 1992; Menon & Vidugiriene, 1994). More than ten genes participate in this biosynthetic pathway. First, the α-GlcNAc is transferred from UDP-GlcNAc to PI to form GlcNAc-PI, which is subsequently de-N-acetylated to GlcN-PI (Doering *et al*., 1989). Then three mannose residues are transferred from Dol-P-Man to GlcN-PI to form Man3GlcN-PI (Menon *et al*., 1990). EtN-P is added to position 6 of the

third mannose from phosphatidylethanolamine, generating NH2-$(CH_2)_2$-PO_4-6Manα1-2Manα1-4Glc-NH_2-myo-inositol-PO_4-lipid. This molecule represents the mature GPI anchor precursor and is transferred to VSG *en bloc*.

1.4 *In vitro* Translation System

1.4.1 General Features of the *in vitro* Translation System

Cell-free protein synthesizing systems have contributed significantly to the understanding of the molecular mechanisms involved in post-transcriptional regulation of gene expression and co- and post-translational modification of proteins. Although a large number of different protein-synthesizing systems have been used, most of the data were collected using rabbit reticulocytes, wheat germ and *E.coli* lysates. There are some other *in vitro* translation system developed from such as *Saccharommyces cerevisiae* (Doering & Schekman, 1997), *Candida albicans* (Colthurst *et al*., 1991), rat hepatocytes (Kimball *et al*., 1989), Hela-cells (Carroll & Lucas-Lenard, 1993) and Crithidia (Cross, 1966; Ilan & Ilan, 1970). The biosynthesis of certain *Trypanosoma brucei* proteins has previously been studied using either reticulocyte lysates (McConnell *et al*., 1981, 1982, 1983; Presper *et al*., 1986) or wheat germ extracts (Mowatt *et al*., 1988; Cornelissen *et al*., 1986). Translation of VSG has been studied in several cell-free systems. In reticulocyte lysates supplemented with dog pancreatic microsomes, VSG containing some N-linked glycans could be produced (Presper & Heath 1986). This system supplemented with trypanosomal microsomes did not result in glycosylation (Moreno & Cross, 1991). In none of these systems GPI anchoring was observed, however. Post-translational modifications of VSG may thus only occur in a homologous cell-free system.

1.4.2 Translation in Trypanosome Lysates

A cell-free system from the bloodstream form of *Trypanosoma brucei* was first demonstrated by Moreno & Cross, 1991. Here VSG synthesized from endogenous mRNA was co-translational N-glycosylated, but reinitiation was rather poor and no signal sequence cleavage or glycosylation did occur. Therefore, we have developed an *in vitro* translation system in our laboratory. However, standard preparation protocols have failed to produce an active *in*

vitro translation system. In fact, the translation activity of reticulocyte lysate could be inhibited by addition of this trypanosome lysates. Through 12 to 24 h dialysis or ultracentrifugation, an endogenous translational active retentate was obtained (Miosga, 1989; Landgraf, 1990). By addition of 2-aminopurine and spermidin, the translation activity was further improved (Bernhagen, 1992). The inhibitor problem was thoroughly solved by Lehner and Hömke (1995, 1997). They applied a method called 'limited lysis'. After 7 min hypotonic homogenization and isotonic adjustment, the lysate was immediately centrifuged (6 min, 12000g, 4°C) to prevent the release of a potent small molecular mass translation inhibitor from lysosomes.

1.5 The Anchor Exchange Enzyme Complex

1.5.1 Supposed Reaction Mechanism

The use of prepromini-placental alkaline phosphatase (MiniPLAP) as substrate led to as much as 10% of the mature product that was cleaved correctly but was not linked to the GPI anchor (Maxwell *et al*., 1995). Some of the factors that influenced the relative proportions of GPI linked to free miniPLAP have been the amount of GPI in the cells and the amino acid substituent at the ω site of the nascent protein. GPI was also necessary even for the formation of free miniPLAP. These results supported a transamidase type of enzyme, which forms an activated intermediate with the carbonyl group of the amino acid at the ω site of pro-peptide. This activated intermediate accepts the nucleophilic amino group of the ethanolamine residue of GPI to form GPI-linked mature miniPLAP or an abundant nucleophile such as water to yield free mature miniPLAP. Direct evidence for a transamidase has been inferred from the finding that a microsomal enzyme activity capable of removing the C-terminal GPI anchor signal was enhanced by small nucleophilic amines (Maxwell *et al*., 1995). The experiments revealed that hydrazine and hydroxylamine, in the presence of rough microsomal membranes, catalyze the conversion of the pro form of miniPLAP to mature form from which the COOH-terminal signal peptide has been removed, apparently at the same site but without the addition of GPI. When a biotinylated derivative of hydrazine was used instead of hydrazine, the released VSG could be precipitated with streptavidin-agarose, indicating that the

biotin moiety was covalently incorporated into the protein. Hydrazine was shown to block the C terminus of the released VSG hydrazide because the released material, unlike a truncated form of VSG lacking a GPI signal sequence, was not susceptible to proteolysis by carboxypeptidases. (Sharma *et al*., 1999) These results showed that the released material is VSG hydrazide and proofed that GPI anchoring proceeds via a transamidation reaction mechanism.

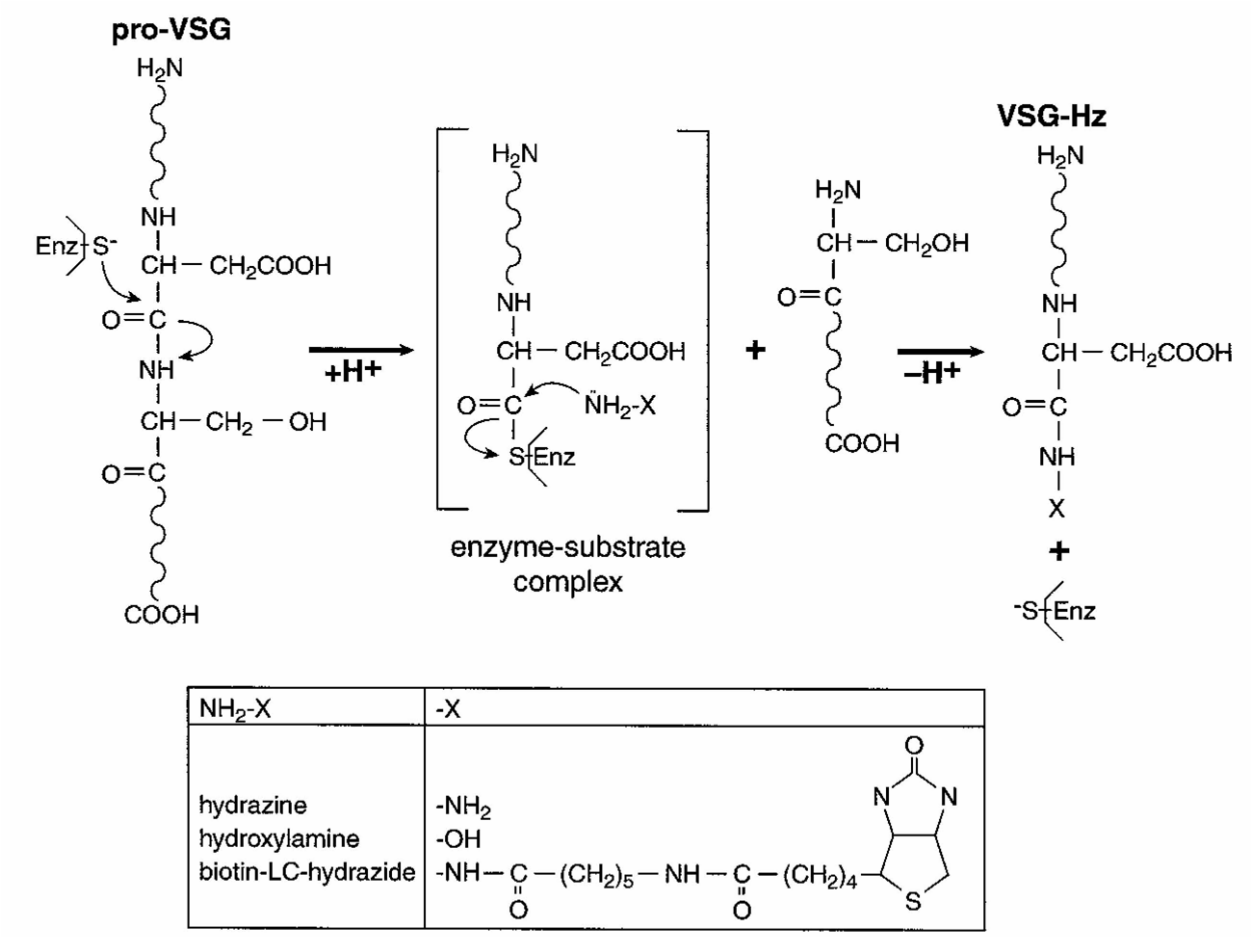

Fig. 1.4: Proposed Mechanism of the Transamidase Mediated Anchor Exchange Reaction
(According to Udenfriend *et al*., 1995 and Sharma *et al*., 1999)

The carbonyl group of the amino acid (aspartic acid) of pro-VSG is activated by a sulfhydryl group in the transamidase (Enz-S) resulting in the formation of an enzyme-substrate complex and cleavage of the amide bond between aspartic acid and serine (ω+ 1 in the c-terminal signal sequence of pro-VSG). Nucleophilic attack by H_2N-X results in release of VSG-NH-X and regeneration of the active site sulfhydryl in the transamidase.

In *Trypanosoma brucei* the reaction could be inhibited with sulfhydryl alkylating reagents, suggesting that the transamidase enzyme contains a functionally important sulfhydryl residue (Mayor *et al*., 1991; Sharma *et al*., 1999).

1.5.2 Gpi8

Genetic approaches have identified genes required for addition of GPI precursor lipids to proteins. Transamidase-deficient cells are expected to accumulate complete GPI lipids as well as GPI precursor proteins. This phenotype is exhibited by two yeast mutants, Gaa1 and Gpi8, and a mammalian mutant cell line (class K) (Mohney *et al*., 1994; Hamburger *et al*., 1995; Benghezal *et al*., 1995; Yu *et al*., 1997; Chen *et al*., 1996). *GPI8* is an essential yeast gene and encodes a type I transmembrane ER protein with a large luminal domain. Yeast Gpi8 has 27.5% identity to jack bean asparaginyl endopeptidase, which shows transpeptidase activity *in vitro* (Abe *et al*., 1993). It belongs to a novel cysteine protease family. Homologies between this family and other Cys proteinases, such as caspases, pointed to Cys199 and His157 as potential active site residues. Indeed, Gpi8 alleles mutated at Cys199 or His157 are nonfunctional, i.e., they are unable to suppress the lethality of Δgpi8 mutants (Meyer *et al.*, 2000). The homology with proteases suggests that Gpi8 is directly involved in the proteolytic removal of the GPI-anchoring signal. The protein contains three N-glycosylation sites. In contrast to Gaa1, which contains KKXX or KXKXX retrieval signals at their cytosolic C-terminal ends, Gpi8 does not contain any known retrieval sequence. Gpi8 may be retained in the ER through an interaction with other ER resident proteins or may contain an unidentified retention motif. It is also possible that Gpi8 is retained in the ER by an interaction with Gaa1 since Gaa1 coprecipitated with Gpi8 (Ohishi *et al*., 2000).

1.5.3 *GAA1* and Other Components

The *GAA*1 gene is also essential and codes for a 68 kDa ER protein with a large luminal domain, several membrane spanning domains and a cytosolic ER retrieval signal on the far end of the C terminus. A *Saccharomyces cerevisiae* gaa1 mutant was defective in posttranslational attachment of GPI to proteins (Hamburger *et al*., 1995). *GAA1* knockout cells were defective in the formation of carbonyl intermediates between precursor proteins and transamidase as

determined by an *in vitro* GPI-anchoring assay. Human and mouse *GAA1* genes consist of twelve exons that span about 4 kb. Human and mouse *GAA1*s are located at 8q24.3 or 15E (chromosome position), respectively (Inoue *et al*, 1999). The amino acid sequence of hGaa1 is 25% identical and 57% homologous to that of yeast Gaa1 (Hiroi *et al*., 1998). Gaa1 and Gpi8 are associated with each other and constitute a GPI transamidase (Ohishi *et al*., 2000). Gaa1 has no homology to other proteins in the databases. This protein is required for the attachment of GPIs to proteins *in vivo* although its exact role is still unknown. Amino acids 311-321 of yeast Gpi8 are essential for GPI attachment. This region is not necessary for association of Gpi8 with Gaa1, however. Therefore, a likely role of this region is to associate with a third component.

1.6 Objective of this Work

The work embodied here envisages different approaches employed for co- and posttranslational processing of VSG. One of the objectives of this work was to use the *in vitro* translation system and *in vitro* culture of the bloodstream form of *Trypanosoma brucei* for the study of N-glycosylation and GPI-anchor exchange. The established cell-free system could incorporate radio labeled amino acids into newly formed proteins with high efficiency similar to reticulocyte lysates. The newly formed VSG was analyzed to testify the processing capability of the *in vitro* translation system. The reaction mechanism was studied using small nucleophilic amines. Although the transfer of preformed GPIs onto proteins can be studied by the *in vitro* translation system, the enzymes involved in this process have not been fully characterized, yet. The GPI-transferase complex is believed to act as a transamidase. Two yeast mutants, Gaa1 and Gpi8, accumulate complete GPIs as well as the GPI precursor proteins. These proteins are required for the attachment of GPIs to proteins. Therefore, *Trypanosoma brucei GPI8* was to be cloned and the recombinant protein should be heterologously expressed in *E. coli* to check for the activity and to generate specific antiserum. This specific antiserum should be used to testify for cell localization of *Tb*Gpi8, Western blotting analysis and immunoprecipitation of the GPI-transferase complex.

2 Materials and Methods

2.1 Materials

2.1.1 General Chemicals

Acrylamide (Rotiphorese Gel 30)	ROTH, Karlsruhe
Agar-Agar	ROTH, Karlsruhe
Agarose	FMC
2-Aminopurine	Sigma, Deisenhofen
Ampicillin	ROTH, Karlsruhe
Amplify™	Amersham Buchler, Braunschweig
Amylose resin	NEB GmbH Schwalbach/Taurus
Albumin, Bovine (BSA)	Sigma, Deisenhofen
Ammonium persulphate (APS)	Merck, Eurolab GmbH Germany
ATP	Sigma, Deisenhofen
UltraLink™Immobilized NeutrAvidin™Plus	Pierce, Illinois, USA
Bisbenzimide	Sigma, Deisenhofen
Bromophenol blue	ROTH, Karlsruhe
Chitin beads	NEB GmbH Schwalbach/Taurus
Chymostatin	Sigma, Deisenhofen
Coomassie brilliant blue G-250	ROTH, Karlsruhe
Creatin phosphate	Sigma, Deisenhofen
DEAE-Sephacel	Sigma, Deisenhofen
Dimethyl sulfoxide (DMSO)	Sigma, Deisenhofen
1kb DNA ladder	NEB GmbH Schwalbach/Taurus
100 bp DNA ladder	NEB GmbH Schwalbach/Taurus
dNTPs	Sigma, Deisenhofen
DTT	ROTH, Karlsruhe
EDTA	ROTH, Karlsruhe
Ethidium bromide	ROTH, Karlsruhe
Formaldehyde (37%)	Sigma, Deisenhofen

Freunds adjuvant, 'complete' and 'incomplete'	Sigma, Deisenhofen
Glycerol (87%)	Merck, Eurolab GmbH Germany
Glycine	Merck, Eurolab GmbH Germany
GTP	Sigma, Deisenhofen
Haemin	Sigma, Deisenhofen
HEPES	Sigma, Deisenhofen
Ni-NTA agarose	Qiagen GmbH, Hilden
Hydrazine-hydrate	Sigma, Deisenhofen
Imidazol	Merck, Eurolab GmbH Germany
Isopropanol	Merck, Eurolab GmbH Germany
L-Isoleucine	Sigma, Deisenhofen
Isopropyl β-D-thiogalactopyranoside (IPTG)	Sigma, Deisenhofen
Leupeptin	Sigma, Deisenhofen
MEM vitamine solution	Sigma, Deisenhofen
Protein molecular marker	Sigma, Deisenhofen
BenchMark™ prestained protein ladder	Gibco BRL Life Technology, Karlsruhe
n-Octyl-D-glucopyranosid	Sigma, Deisenhofen
Pepstatin A	Sigma, Deisenhofen
Pfu-Polymerase buffer	Stratagene Europe Netherland
Phenol	ROTH, Karlsruhe
Polyethylene glycol 6000 (PEG 6000)	ROTH, Karlsruhe
Restriction enzyme buffer	NEB GmbH Schwalbach/Taurus
RNasin®ribonuclease inhibitor, 40U/ml	Promega, Madison, USA
Sepharose 4B, CNBr-activated	Sigma, Deisenhofen
Sodium dodecyl sulfate (SDS)	Serva, Heidelberg
SuperSignal® West Pico Chemiluminescent substrate	Pierce, Illinois, USA
Taq-polymerase buffer	Sigma, Deisenhofen
T4-ligase buffer	Gibco BRL Life Technology, Karlsruhe
TEMED	ROTH, Karlsruhe
Tris-hydroxymethyl-aminomethane (Tris)	Serva, Heidelberg
Triton X-100	Sigma, Deisenhofen

Tunicamycin	Sigma, Deisenhofen
Tween 20	ROTH, Karlsruhe
Ultima Gold^{TM} Scintillation cocktail	Packard, Frankfurt a.M.
Urea	Merck, Eurolab GmbH Germany
Yeast extract	ROTH, Karlsruhe
$ZnCl_2$	Merck, Eurolab GmbH Germany

2.1.2 Radiochemicals

L-[^{35}S]-methionine	ICN, Eschwege
UDP-[^{14}C]-galactose	Amersham Buchler, Braunschweig

2.1.3 Kits

Agarose gel elution kit	Roche Boehringer Mannheim GmbH
ECL^{TM} *in vitro* translation system	Amersham Buchler, Braunschweig
Biotin-lysine-tRNA^{lys} Set	Roche Boehringer Mannheim GmbH
IgY purification kit	ACROS Organics, Belgien

2.1.4 Enzymes and Antibodies

alk. phosphatase (CIP)	NEB GmbH Schwalbach/Taurus
*Eco*RI	NEB GmbH Schwalbach/Taurus
*Eco*RV	NEB GmbH Schwalbach/Taurus
Enterokinase	Roche Böhringer Mannheim GmbH
*Kpn*I	NEB GmbH Schwalbach/Taurus
Creatinkinase	Serva, Heidelberg
*Nde*I	NEB GmbH Schwalbach/Taurus
N-glycosidase F (PNGase F)	Roche Böhringer Mannheim GmbH
*Nhe*I	NEB GmbH Schwalbach/Taurus
Nuclease S7 from *Staphylociccus aureus*	Roche Böhringer Mannheim GmbH
Pfu-polymerase	Stratagene Europe Netherland
Polynucleotidkinase	NEB GmbH Schwalbach/Taurus

RNase A	Sigma, Deisenhofen
*Sap*I	NEB GmbH Schwalbach/Taurus
Taq-polymerase	Sigma, Deisenhofen
T4 polynucleotide kinase	NEB GmbH Schwalbach/Taurus
T4-DNA-ligase	NEB GmbH Schwalbach/Taurus
*Xho*I	NEB GmbH Schwalbach/Taurus
Anti-rabbit-IgG peroxidase conjugate	Sigma, Deisenhofen
Anti-rabbit-IgG FITC conjugate	Sigma, Deisenhofen
Anti-rabbit-IgG gold conjugate	Sigma, Deisenhofen
Anti-mouse-IgG peroxidase conjugate	Sigma, Deisenhofen
Anti-mouse-IgG FITC conjugate	Sigma, Deisenhofen
Anti-chicken IgG peroxidase conjugate	Sigma, Deisenhofen
Anti-chicken IgG FITC conjugate	Sigma, Deisenhofen
Anti-chicken IgG gold conjugate	Sigma, Deisenhofen
Anti-CRD serum from rabbit	gift of T. Ilg (MPI for Biology, Tübingen, Germany)
Anti-MBP serum from rabbit	Sigma, Deisenhofen

2.1.5 Equipments

Centrifuges

Biofuge A	Heraeus, Osterode
Microfuge E	Beckmann, München
Sigma 302K Labor Centrifuge	Sigma, Deisenhofen
Suprafuge 22 with Rotor HFA 22.50	Heraeus, Osterode
Table Centrifuge Denver Force 13	Denver Instrument Company, Arvada, USA

Electrophoresis and Western Blotting

Electrophoresis Minigel System 'Mighty Small II' SE 250	Hoefer Scientific Instrument, San Francisco, USA
Semi-Dry Eletrophoretic transfer Cell	BioRad, München

LSC-Analysis

Filtration Apparatus 1225 Sampling Manifold	Millipore, Bedford, USA
Liquid Scintillation Counter LSC 1600 CA	Packard, Frankfurt a.M

2.1.6 Experimental Organisms

Trypanosoma b. brucei

MITat 1.2	221 of strain EATRO 427
MITat 1.4	117 of strain EATRO 427
MITat 1.5	118 of strain EATRO 427

E. coli

XL-1 Blue	Stratagene Europe Netherland
ER2566	NEB GmbH Schwalbach/Taurus

2.1.7 Other Consumable Materials

BioMax MR-1 *scientific imaging film*	Kodak, Stuttgart
BioMax Light-1 *scientific imaging film*	Kodak, Stuttgart
Dialysis tube	Serva, Heidelberg
Glasfaser filter GF/C	Whatman, Maidstone, USA
HybondECL™ nitrocellulose membrane	Amersham, Braunschweig
Microcon 30 ultrafiltration units	Amicon, Witten
Microplate	Nunc, Denmark
Object plate for immunofluorescence	bioMĕrieux, France
Parafilm	American Can Co., Chicago
Sterile tube, Falcon®, 15ml, 50ml	Becton Dickingson, New Jersey
Whatman 3MM paper	Whatman, Maidstone, USA
Sterile filter SFCA-membrane	Nalge, Hereford (UK)

2.1.8 Vectors and Primers

Vectors

pBS KS^+	Stratagene Europe Netherland
pTYB1	NEB GmbH Schwalbach/Taurus
$pMAL^{TM}$-c2	NEB GmbH Schwalbach/Taurus
pRSET B	Invitrogen, Karlsruhe
pEGFP-N1	Clontech, California, USA

Primers	**Sequence**
Cloning of GPI8	
GPI8AS1	5'-CCACATCATCATIAGIGTITCIGCIATITC-3'
SLSE23	5'-CGCTATTATTAGAACAGTTTCTG-3'
GPI8SE3	5'-GGACTCGGAGTTCATGAGCTC-3'
OT203N	5'-CCCGGGT_{20}VNN-3'
GPI8SE1	5'-CGCAGAGGTTTCAAACAAGTGG-3'
GPI8AS2	5'-CTTTGTTGCACGTGACTACAATA-3'
Maltose expression system for GPI8	
GPSEMAL1	5'-GCAGCAGGTACCGGCGGAAGGCTTTCATGGTATG-3'
GPASMAL2	5'-GATATAGGTACCCTAGAACAAATCGTAACGTAACTCTAC-3'
His-tag expression system for GPI8	
GPI8-His-SE2	5'- GCTGCAGCTAGCATGAATAAAACCAACACC TGG-3'
GPI8-AS	5'-CGAGTAGAATTCCTAGAACAAATCGTAACGTAACTC-3'
Intein expression system for GFP-221VSG	
221-PEPSE	5'-GATATACATATGCTGGAGGGCAATGCCAAGTTAACGACTATAC-3'
221-PEPAS	5'-AAGCCAAAGAGGGGTCTTGCT-3'
3GFP-NDE	5'-CGCTACATATGCTTGTACAGCTCGTCCATGCCGAG-3'
5GFP-NDE	5'-CGCTACATATGGTGAGCAAGGGCGAGGAGCTG-3'

Further Oligonucleotides

221-5ADA	p-GCAGTTTTGCTTTTT
221-3ADA	p-GCAAAAAAGCAAAACTGC

2.2 Media, Buffers and Solutions

2.2.1 Cultivation and Isolation of Trypanosomes

Citrat-Glucose-Anticoagulant (CGA) pH 7.7

Na_3-citrate x $2H_2O$	100 mM
Glucose	40 mM

Separation buffer, pH 8.0 (Lanham & Godfrey, 1970)

Na_2HPO_4 x $2H_2O$	57 mM
KH_2PO_4	3 mM
NaCl	44 mM
Glucose	55 mM

Basic medium, pH 7.4

(According to Eagle, 1959; modified by Duszenko *et al*., 1985, 1992)

	[mg/l]
$CaCl_2$ x H_2O	265
KCl	400
$MgSO_4$ x $7H_2O$	200
NaH_2PO_4 x H_2O	140
NaCl	6800
HEPES	7140
Phenol Red	10

These chemicals were dissolved in water and the pH was adjusted to 7.4.

L-Arginine/HCl	126
L-Cysteine	24
L-Histidine/HCl x H_2O	42
L-Isoleucine	52
L-Leucine	52

L-Lysine	73
L-Methionine	15
L-Phenylalanine	100
L-Threonine	48
L-Tryptophan	10
L-Tyrosine	100
L-Valine	46
L-Ornithine/HCl	10
Adenosine	12

These amino acids and adenosine were dissolved separatly, mixed together and then added to following stock solutions:

	[ml/l]
MEM nonessential amino acids solution	10
MEM vitamine solution	10

This so-called basic medium was sterilized and could be stored at 4°C for up to 3 months.

Complete culture medium

For preparation of a complete culture medium the following stock solutions were added to the basic medium:

		[ml/l]
$NaHCO_3$	(750mg/10ml distilled water)	30
L-Cysteine	(30.3mg/10ml distilled water)	10
Bathocuproindi-sulfonate (BCS)	(5.6mg/10ml distilled water)	1
Hypoxanthine	(13.6mg/10ml 0.1 N NaOH)	10
2'-Desoxythymidine	(3.9mg/10ml distilled water)	10
L-Glutamine	(292mg/10ml distilled water)	10
Sodiumpyruvate	(220mg/10ml distilled water)	10
Myristic acid linked on defatted BSA (50 x)		20
Fetal calf serum	(heat inactivated: 30min. 56°C)	100
Penicillin-Streptomycin-solution		5

Preparation of the myristic acid linked to defatted BSA (Ferguson & Cross, 1984):

24 mg myristic acid was dissolved in 100 µl ethanol (95%). This myristic acid was then gradually added to a BSA solution (1 g defatted BSA/20ml distilled water). The medium was sterilized and stored at 4°C for about 4 weeks.

Culture medium for procyclics pH 7.4

The following stock solutions were added to the basic medium to cultivate of procyclic parasites:

		[ml/l]
Glutamine	(5.6mg/10ml distilled water)	10
Haemin	(25mg/10ml distilled water)	3
Pyruvate	(220mg/10ml distilled water)	10
Proline	(600mg/10ml distilled water)	10
Fetal calf serum	(heat inactivated: 30min. 56°C)	100
Penicillin-Streptomycin-solution		5

The culture medium was sterilized and stored at 4°C for up to 4 weeks.

Tunicamycin stock solution

250 µg/ml in H_2O (25x)

2.2.2 Lysates Preparation from *T. brucei* for Cell-Free Translation

Protease inhibitor mix (1000 x)

Pepstatin	1 mM
Chymostatin	1 mM
Leupeotin	1 mM

The 3 inhibitors were dissolved in DMSO and stored as aliquots at -20°C.

2-Aminopurin stock solution

2-Aminopurin	500 mM

dissolved in DMSO.

Lysis medium

DTT	2 mM
Protease inhibitors mix	1 µM
2-Aminopurin	10 mM

Phosphate buffer (PB), pH 7.4 (10 x)

Na_2HPO_4	138.2 mM
KH_2PO_4	42.7 mM

2.2.3 RNA Isolation from Trypanosomes

Chloroform/isoamylethanol	24:1

TM-Buffer, pH 8.6

Tris/HCl	50 mM
$MgCl_2$	5 mM

TE-Buffer, pH 7.5

Tris/HCl	10 mM
EDTA	1 mM

DNase-Buffer, pH 7.5

Tris/HCl	40 mM
$MgCl_2$	6 mM

2.2.4 *In vitro* Translation

Multi supplement

Amino acids without methionine	each 1 mM
DTT	21.8 mM
Spermidine chloride	2.1 mM
Peotease inhibitors	each 10 µM
$MgCl_2$	21.3 mM
KAc	1.7 mM

Stored at -70°C.

Creatinkinase solution

Creatinkinase	200U/ml
KCl	100 mM
HEPES-KOH, pH 7.4	20 mM
Glycerol	50 % (v/v)

Stored at -20°C.

Standard supplement mix

Multisupplement (stock solution, -70°C)	94 µl
Creatinkinase solution (stock solution, -20°C)	20 µl

1 M Creatinphophate	10 µl
10 mM GTP (stock solution, -70°C)	15 µl
100 mM ATP	10 µl

Prepared immediately before each use.

L-[^{35}S]-methionine *in vitro* translation mix

Supplement mix without methionine	6 µl
Lysate	20 µl
[^{35}S]-methionine	300 kBq
Distilled water	*ad* 40 µl

Biotin *in vitro* translation mix

Supplement mix (without amino acids)	6 µl
Amino acids without lysine	1.25 nmol
Lysate	20 µl
Biotin-lys-tRNA	10 pmol
Distilled water	*ad* 40 µl

2.2.5 SDS-PAGE According to Laemmli

Running gel (2.6% C)	(10% T)	(12% T)
Rotiphorese gel 30	15.2 ml	18.24
1 M Tris-HCl pH 8.8	17.2 ml	17.2
Distilled water	13.2 ml	10.16
10% SDS	456 µl	456 µl
10% Ammonium peroxodisulfat	156 µl	156 µl
TEMED	48 µl	48 µl

Stacking gel

Rotiphorese Gel 30	4.0 ml
1 M Tris-HCl pH 6.8	3.0 ml
Distilled water	16.8 ml
10% SDS	240 µl
10% Ammonium peroxodisulfat	120 µl
TEMED	24 µl

Sample buffer (2 x)

Tris-HCl pH 6.8	180 mM
SDS	4.4 %
Glycerol	19.4 %

DTT/BPB (20 x)

DTT	1 M
Bromophenolblue	0.2 %

Running buffer

Tris/HCl	25 mM
Glycine	192 mM
SDS	0.1 %

Coomassie Staining

Staining solution

Coomassie Brilliant Blue R	0.1 %
Methanol	40 % (v/v)
Acetic acid	10 % (v/v)

Destaining solution

Acetic acid	10 % (v/v)
Methanol	40 % (v/v)

Silver Staining

Fixative

Acetic acid	24 ml
Ethanol	80 ml
Formaldehyde 37 %	100 µl
Distilled water	*ad* 200 ml

Wash solution

Ethanol	50 %

Incubation solution

10 % $Na_2S_2CO_3$	400 µl
Distilled water	*ad* 200 ml

Silver stain

Silver nitrate	0.4 g
Formaldehyde (37 %)	150 µl
Distilled water	*ad* 200 ml

Developing solution

Na_2CO_3	18 g
10 % $Na_2S_2CO_3$	12 µl
Formaldehyde (37 %)	150 µl
Distilled water	*ad* 300 ml

Stop solution

Acetic acid	24 ml
Ethanol	80 ml
Distilled water	*ad* 200 ml

Gel drying solution

Ethanol	25 % (v/v)
Glycerol	2 % (v/v)

2.2.6 Western-Blotting

Transfer buffer, pH 9.2 (Bjerrum & Schafer-Nielsen, 1986)

Tris/HCl	48 mM
Glycine	39 mM
SDS	0.00375 %
Methanol	20 %

Blocking buffer

Defatted milk power in PBST	10 %

PBS

Na_2HPO_4	80 mM
NaH_2PO_4	20 mM
NaCl	100 mM

PBS-Tween

Tween 20 in PBS	0.1 %

Stripping buffer, pH 6.7

2-mercaptoethanol	100 mM
SDS	2 %
Tris-HCl	62.5 mM

2.2.7 Deglycosylation Using N-Glycosidase F

Deglycosylation buffer, pH 7.5

Na_2HPO_4/NaH_2PO_4	250 mM
EDTA	10 mM
N-Octylglucosid	1.4 %
β-Mercaptoethanol	1 mM
NaN_3	0.05 %
Protease inhibitors	1 µM each

2.2.8 Bacterial Media

LB-medium, pH 7.0

Peptone	1 %
Yeast extract	0.5 %
NaCl	0.5 %

Adjusted to pH 7.0 using 1 M NaOH.

Ampicillin stock solution (1000 x)

Ampicillin in 70 % ethanol	50 mg/ml

Culture plate

Agar-Agar in LB medium	1.5 %

Transformation medium for *E.coli* (TSS-Buffer)

DMSO	5 %
$MgCl_2$	50 mM

PEG6000	10 %

made from LB medium.

2.2.9 Molecular Biology

DNA sample buffer, pH 8.0

Glycerol	5 %
Bromophenol blue	0.025 %
EDTA	0.1 M

TAE-buffer, pH 8.0

Tris-HCl	0.04 M
EDTA	0.001 M

Adjusted to pH 8.0 with acetic acid.

2.2.10 Heterologous Expression and Purification of Recombinant Protein

pMAL™ System

Column buffer and lysis buffer, pH 7.4

Tris-HCl	20 mM
NaCl	200 mM
EDTA	1 mM

Elution buffer

Maltose in column buffer	10 mM

Incubation buffer for enterokinase, pH 8.0

Tris-HCl	50 mM

IMPACT™ System

Lysis buffer, pH 8.0

HEPES	20 mM
NaCl	500 mM
EDTA	1 mM

Triton X-100	0.1 %

Column buffer, pH 8.0

HEPES	20 mM
NaCl	500 mM
EDTA	1 mM

Cleavage buffer, pH 8.0

HEPES	20 mM
NaCl	500 mM
EDTA	1 mM
DTT	50 mM

Stripping buffer, pH 8.0

HEPES	20 mM
NaCl	500 mM
SDS	1 %

6xHis Tag System

For Native Conditions

Lysis buffer, pH 8.0

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	10 mM

Wash buffer, pH 8.0

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	20 mM

Elution buffer, pH 8.0

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	250 mM

For Denaturing Conditions

Lysis buffer, pH 8.0

NaH_2PO_4	100 mM
Tris-HCl	10 mM
Urea	8 M

Wash buffer, pH 6.3

NaH_2PO_4	100 mM
Tris-HCl	10 mM
Urea	8 M

Elution buffer, pH 4.5

NaH_2PO_4	100 mM
Tris-HCl	10 mM
Urea	8 M

2.2.11 Detection of the Transamidase Activity of Trypanosomal Lysate and Cloned *Tb*Gpi8 Using Fluorogenic Peptide Substrate

Detection buffer, pH 6.5

Hepes	50 mM
NP40	0.1%
Protein inhibitors	1 µM each

2.2.12 Production of Antibodies

Immunization emulsion C

MBP-Gpi8	0.5 mg
PBS	500 µl
Freunds *complete* adjuvant	500 µl

Immunization emulsion I

MBP-Gpi8	0.5 mg
PBS	500 µl
Freunds *incomplete* adjuvant	500 µl

MBP-*Tb*Gpi8 in PBS was emulsified with adjuvant.

2.2.13 Isolation and Affinity Purification of Special Antibodies

Phosphate buffer, pH 7.2

Na_2HPO_4	1.8 g/l
KH_2PO_4	1.4 g/l
NaCl	5.8 g/l

PEG 6000

PEG 6000 in phosphate buffer	7 %, 24 % and 50 %

Elution buffer pH 2.5

Glycine/HCl	100 mM

2.2.14 Immunofluorescence

Hank's buffered saline (HBS) pH 7.3

NaCl	0.85 %
HEPES	25 mM

FHBS

Formaldehyde in HBS	1 %

2.2.15 Electron Microscopy

EM-buffer, pH 7.4 (Glaubert, 1975; Langreth & Balber, 1975)

Sodium cacodylate x 3 H_2O	0.1 M
Sucrose	0.12 M

Glutaraldehyde solution (Glaubert, 1975)

Glutaraldehyde in EM-buffer	2 %

Uranyl acetate solution

Uranyl acetate in distilled water	5 %

Stored at RT in the dark.

Osmiumtetroxide solution

Osmiumtetroxide in distilled water	3 %

Epon-Mix (Luft, 1961)

Epon 812	16.8 g
DDSA	8.0 g
MNA	8.7 g
DMP-30	0.3 g

Should be prepared immediately before use.

2.2.16 Immunoprecipitation

Precipitation buffer, pH7.8

Tris-HCl	50 mM
NaCl	150 mM
NP-40	1 %
Sodium azide	0.025 %
Protein inhibitors	1 µl each

2.3 Methods

2.3.1 General Methods

2.3.1.1 SDS-PAGE According to Laemmli

SDS-PAGE was accomplished in Mighty Small II SE 250 gel chambers according to the method of Laemmli (Laemmli, 1970) with 4 % stacking gel and 10% or 12 % running gel. For determination of the molecular mass scale suitable molecular markers were used.

2.3.1.2 Commassie Staining

Following electrophoresis, the gel was stained in 1 mg/ml Commassie blue R for at least 1 h. The gel was destained in destaining solution for about 2 h.

2.3.1.3 Silver Staining

The gel was fixed in fixative for 10 min and washed with ethanol (50 %) 3 times for 10 min each. After incubated 1 min in incubation buffer, the gel was washed with water 3 times for 20 sec. The gel was then incubated in silver stain solution for 20 min and washed 2 min with water. Thereafter the gel was soaked in developing solution until bands appeared. The gel was treated with stop solution and washed with 50 % ethanol.

2.3.1.4 Fluorography

After Coomassie- or Silver staining, the gel was incubated in Amplify^{TM} for 15-30 min. The dried gel was exposed to Biomax MR (Kodak) film at -70°C. The film then was developed and fixed. The exposure time varied according to the intensity of radioactivity between 2 days and 1 week.

2.3.1.5 Western Blotting Analysis

Western blotting was performed using a *Semi-Dry*-Apparatus. Three filter papers were laid on the anode plate. Then nitrocellulose membrane, gel and 3 filter papers were added one after another. All the materials were soaked with transfer buffer before use. The electro transfer was carried out by a constant electric current of 5.5 mA/cm^2 for 30 min.

2.3.1.6 Immunofluoroscopy of Cells

1 x 10^7 trypanosomes were fixed in 1 ml FHBS for at least 1 h. After two times washing with cold HBS and two times washing with cold 1 % BSA cells were resuspended in 400 ul 1 % BSA. 5 µl of this suspension was transferred to glass slides and dried at 37°C for 3 h or at RT overnight. The slide with fixed cells was incubated 15 min with 10 µl first antibody in a wet box and washed 5 min with distilled water and dried at RT. It was then incubated 15 min with FITC labelled second antibody and washed again for 5 min. The slide was then incubated in bisbenzimid for 15 min and again dried at RT. The photography document was viewed and analyzed using an Olympus BH2 fluororesent microscope and Biosis imaging software.

2.3.1.7 Electron Microscopy of Cells

Fixation

1 x 10^8 trypanosomes were fixed in 2% glutaraldehyde in EM-buffer at 4°C for 60 min (Langreth & Balber, 1975; Glauert, 1975). The cells were washed two times with EM buffer for 5 min and stained with 1.5% osmium tetroxide at 4°C for 60 min. The cells were washed two times with EM buffer and two times with distilled water (5 min each). For additional staining, the cells were incubated with 0.5% uranyl acetate at RT for 60 min and washed with distilled water. The fixed cells were dehydrated using ethanol solutions of increasing concentrations (30%, 50%, 70%, 95%, 100%, 100%, 1 h each at RT) and washed two times 10 min each with propylenoxide.

Embedding

The sample was incubated with Epon:propylenoxide (1:1, 60 min, RT), infiltrated with pure Epon (60 min, RT) and transferred to Beem^R-Cups. The polymerization was carried out for 12 h at 45°C and 36 h at 60°C.

Ultra sectioning

After polymerization the tip of the sample was trimmed to trapezoidal shape on Primetime 11800, sectioned using an Ultra tome and fished with copper grits.

Immunogold labeling

The grit was washed 3 times with 20 mM Glycine/PBS and incubated with 10 µl of the first antibody (1:100) (60 min, RT). After 3 times washing with 0.1% BSA/PBS the sample was incubated with 10 µl of second antibody for 60 min. The grit was then washed 3 times with 0.1% BSA/PBS and 5 times with distilled water.

Contrasting

The grit was contrasted on a 50 µl 5% uranyl acetate drop (60 min, RT, face down) (Lewis & Knight, 1977). The grit was washed with distilled water and incubated in Reynolds lead citrate solution (7 min, RT) and again washed and dried.

Microscopy

The sample was viewed and photographed on Zeiss EM 109 transmission electron microscope with TFP-camera using negative film Kodak Plus-X-Plan 120 (125 ASA, 60 x 70 mm).

2.3.2 *In vitro* Translation System

2.3.2.1 Cultivation and Isolation of Trypanosomes from Rat

Long slender bloodstream forms of *T. b. brucei* were grown in rats. Rats were injected with 2-4 x 10^7 trypanosomes intraperitoneally in a volume of about 500 µl. After 2.5 days, trypanosomes grew to about 0.5-1 x 10^9 cells/ml. Normally they have 6 hours generation time. If the cell concentration was above 5 x 10^8/ml, the rats were killed with CO_2. The blood was collected in the thorax by cutting the *Vena cava inferior*. Some CGA (~1 ml) was given to the thoracic cavity and the centrifuge tube to prevent blood coagulation. Infected blood was centrifuged at 1,300 g for 10 min at 4°C and the *buffy coat*, which contained the trypanosomes, was resuspended in separation buffer. The *buffy coat* was applied to a column with about 70 ml DEAE-cellulose and washed with separation buffer immediately. The flow-through containing the trypanosomes was collected and spun again as before.

2.3.2.2 Lysis of Trypanosomes

The freshly isolated trypanosomes were lysed in cold lysis buffer at a cell density of 7x10^9/ml and immediately homogenized with a Dounce-Homogenisator. The cell lysis was controlled by phase microscopy. After cell lysis 1/9 volume phosphate buffer (PB, 10x) was added to the lysate. The lysate was centrifuged at 12,000 g for 6 min at 4°C to remove cell debris and the supernatant was immediately aliquot and stored in liquid nitrogen.

2.3.2.3 *In vitro* Translation Using Trypanosomal Lysates

The cell free system was prepared from trypanosomal lysates (final concentration 50%, v/v) that was supplemented with standard supplement mix, distilled water and [^{35}S]-methionine or biotin-lys-tRNA^{lys} and UDP-[^{14}C]-galactose. The translation mix contained:

Cell lysate	PMS (postmitochondr. supernatant)	50 % (v/v)
Amino acids	19 proteinogenic amino acids without Methionine or lysine respectively	100 uM
Labelled substrate	[^{35}S]-methionine	7.5 kBq/µl
	or biotin-lys-tRNA^{lys}	0.25 pmol/µl
	UDP-[^{14}C]-galactose	46 Bq/µl
Energy regenerating system	ATP	1.0 mM
	GTP	150 µM
	Creatine phosphate	10 mM
	Creatine kinase	4 U/ml
Buffer	Na_2HPO_4	6.9 mM
	KH_2PO_4	2.1 mM
	HEPES	403 µM
Electrolyte	KAc	161 mM
	KCl	2 mM
	$MgCl_2$	2 mM
Polyamine	Spermidinchlorid	200 µM
Protectors and Inhibitors	DTT	3.0 mM
	2-Mercaptoethanol	0.2-0.8 mM
	Pepstatin A, chymostatin, leupeptin	each 1 µM
	2-Aminopurin	4.5 mM
	$ZnCl_2$[1]	0.2 mM
Other components[2]	Glycerin	1 % (v/v)
	DMSO	1 % (v/v)
	Kalium-pyridin-3,4-dicaboxylate	0.3-0.8 mM

1 Used to inhibit GPI-PLC.

2 These components were added from stock solutions.

The mix was incubated at 30°C for 60 min and cooled on ice thereafter to stop the reaction.

2.3.2.4 LSC Quantification of the Radioactive Incorporation

After *in vitro* translation 450 µl 1 N NaOH and 50 µl H_2O_2 were added to 5 µl lysate sample and incubated at 37°C for 30 min. Thereby tRNA linked [^{35}S]-methionine was released and would not be co-precipitated with protein in the next step. For precipitation of the protein 20 µl 1 % BSA and 1.5 ml 25 % TCA were added prior to the incubation on ice water for at least 6 hours or over night. Glass fibre filter GF/C was placed on a filtration apparatus and was socked in 8 % TCA. The precipitated material was resuspended with a plastic pipette and transferred onto the filter. The filter was then washed with 2.5 ml 8 % TCA. Additionally each filter was washed 3 times with 8 % TCA and 1 time with ethanol. The filter was dried by air pumping 1-3 min. The activity on the filter was measured in scintillations-cocktail Ultima Gold™ by *liquid scintillation counting* (LSC).

2.3.2.5 N-Glycosidase F Treatment after *in vitro* Translation

SDS was added to the translation products to give a 2 % concentration. The samples were boiled for 5 min and cooled on ice. 20 µl of this material was added to 170 µl incubation buffer (see 2.1.9.6) and again boiled for 5 min. After cooling the probes to 37°C, 2 U N-glycosidase F was added and incubated at 37 °C for 20 hours. After incubation, the sample was diluted to 1 ml and mixed with 5 µl cytochrome C and 8 µl sodium desoxycholate and incubated at room temperature for 15 min. After mixing with 100 µl TCA (67 %) the sample was centrifuged at 10,000 g for 30 min at 4°C. The pellet was treated with 20 µl SDS-PAGE loading buffer and 8 % NH_4OH was added until the color changed from orange to blue.

2.3.2.6 Capture of Biotinylated Translation Products

60 µl translation mix was diluted in 0.5 ml of PBS-Tween. 100 µl of avidin agarose were added to the mix and the reaction tube was incubated by end-over-end rotation at 4°C for 1 h. After 3 washing cycles (5 min each in 1 ml of PBS-Tween on a rotary mixer at 4°C, followed by a 1 min centrifugation in a

microfuge) the pellet was resuspended in 40 µl of SDS-PAGE loading buffer. The biotinylated proteins were eluted by heating the tube to 100°C for 4 min.

2.3.2.7 Detection and Characterization of the Biotinylated Translation Products

After electrophoresis, the proteins were blotted onto a nitrocellulose membrane. The membranes were probed with streptavidin-horseradish peroxidase for detection of the newly formed protein, with clone-specific antibodies for detection of VSG or with antibody specific for the CRD for detection of the GPI anchor respectively. The results were visualized with ECL™ detection reagents and by fluorography.

2.3.3 *In vitro* Cultivation of Trypanosomes

2.3.3.1 Cultivation of Bloodstream Form Trypanosomes *in vitro*

Cells were cultivated at 37°C in a water saturated atmosphere containing 5 % CO_2. The medium was incubated at 37°C before used. Cells were harvested at a density of about 1-2 x 10^6/ml. For glycosylation inhibition and anchor exchange either 10 µg/ml tunicamycin and/or 0.5 mM hydrazine was added to the culture medium.

2.3.3.2 Cultivation of Procyclic Form Trypanosomes *in vitro*

The cells were cultivated at 27°C with medium in an incubator without CO_2. Cells harvested at a density of about 2 x 10^6/ml.

2.3.4 Cloning

2.3.4.1 PCR

Standard-PCR using Taq-polymerase

PCR reactions were performed in 50 µl reaction mixture containing 50 ng template DNA, 5 pM of each primer, 0.2 mM dNTPs and 2.5 units *Taq*-DNA polymerase in PCR *Taq*-polymerase buffer. The reaction was cycled though the

following temperature profile, preceded by an unique 3 min denaturation step at 94°C:

Denaturation	1 min, 94°C
Annealing	1 min, 45°C
DNA synthesis	1 min, 72°C
No of cycles	30

Control-PCR using Taq-polymerase

These PCR reactions were also performed in a volume of 50 µl containing 5 pM of each primer, 0.2 mM dNTPs in *Taq*-polymerase buffer and 2.5 units *Taq*-DNA polymerase. The templates were either plasmids from mini-preparation (1:1000 diluted) or cultured cells alone. The reaction was cycled though the following temperature profile:

Denaturation	1 min, 94°C
Annealing	45 sec, 38°C
DNA synthesis	1 min, 72°C
No of cycles	30

PCR-using proof reading polymerase

PCR reactions were performed in 50 µl reaction mixture containing 50 ng template DNA, 5 pM of each primer, 0.2 mM dNTPs and 1.25 units *Pfu*-DNA polymerase in PCR *Pfu*-polymerase buffer. PCR *Pfu*-polymerase should be the last component added to the PCR mixture. The PCR-mixture was prepared at 4°C. The reaction was cycled though the following temperature profile:

Denaturation	1 min, 94°C
Annealing	1 min, 45°C
DNA synthesis	2 min, 72°C
No of cycles	30

2.3.4.2 Agarose Gel Electrophoresis

The DNA fragments were separated in submersed horizontal agarose gels (0.8%) with 0.5 µg/ml ethidium bromide. The samples were mixed with loading solution and were electrophoresed along with size markers. The electrophoresis

was carried out in TAE buffer at 60-100 kV for 30 min and DNA was visualized under UV light.

2.3.4.3 Elution of DNA from Agarose Gel

The ethidium bromide stained agarose gels were inspected under UV light and the part of the gel that contained the desired DNA fragment was excised out using a clean sharp scalpel. The gel slice was transferred to a 1.5 ml microfuge tube and buffer 1 of kit (agarose gel elution kit) was added to dissolved agarose and incubated at 50°C for 5 min. After complete solubilization of agarose, 10 µl of the resuspended solution 2 was added and incubated at 50°C for 10 min by mixing the reaction vial every 2 min. The silica particles were precipitated by a brief centrifugation (30 sec.) and the supernatant was removed carefully. The pellet was resuspended in 500 µl buffer 3 by vortexing and then recentrifuged. Then pellet was air-dried for 10-15 min until it turned to white. The DNA was eluted by resuspending the pellet in 20 µl 10 mM Tris-HCl, pH 7.5 and subsequent incubation at RT for 5 min. The elute was separated from the silica gel by centrifugation and was transferred to a fresh vial.

2.3.4.4 Isolation of Plasmid from Bacteria (Minipreparation)

1 ml overnight grown bacterial cultures were concentrated by centrifugation (13,000 g, 30 sec) in Eppendorf tubes. Cells were resuspended in 150 µl TGE (pH8.0) and lysed by addition of 300 µl lysis solution (200 mM NaOH, 1% SDS, freshly prepared) and incubated for 2 min at RT. Thereafter 450 µl chilled KAc (5M, pH 4.8) was added and mixed carefully. After 5 min incubation on ice, the cloudy and viscous solution was centrifuged (10 min, 13,000 g) and the supernatant was collected. This processure was repeated one time again. The collected supernatants were mixed with isopropanol (0.7-1 x v), vortexed and centrifuged (13,000g, 15 min, 4°C). The supernatant was discarded and the pellet was washed with 70% ethanol (-20°C). The pellet was then dissolved in 50 µl Tris-HCl (pH 7.5) containing 10 µg/ml RNase A.

2.3.4.5 Restriction Digestion of DNA

Plasmid DNA and PCR products were digested with endonucleases and checked by gel electrophoresis. Approximately 100 ng - 3 µg DNA in 20-50 µl was incubated with endonucleases for 1-2 h at 37°C using producer's buffer

according to the manufacturer's instruction. The RNA in Plasmid DNA solution from minipreparation was removed by incubation with 1/20 vol RNAase A solution (10 mg/ml) along with restriction enzymes.

2.3.4.6 Ligation

The restriction digested PCR products and plasmids were prepared for ligation by phosphorylation and dephosphorylation using T4 polynucleotide kinase and calf intestine alkaline phosphatase (CIP). The T4 DNA ligase and ligase buffers were added according to the instruction of the producers. Vector and insert DNA were used in the ratio of 1 to 2.5.

2.3.4.7 Preparation of Competent Bacterial Cells

A single colony of *E. coli* (strains XL1-Blue or ER2668) was grown at 37°C in LB medium until the OD_{600} reached about 0.5. Cells was harvested by centrifugation (5,000 g, 10 min, 4°C) and resuspended in 10 ml (1/10 vol.) ice-cold TSS-buffer by gentle shaking (5% DMSO, 50 mM $MgCl_2$, 10 % PEG6000) (Chung *et al*., 1989). The competent cells were aliquoted (100 µl) shock frozen in liquid nitrogen and stored at -80°C until used for transformation with recombinant plasmid DNA.

2.3.4.8 Transformation

5-10 µl of the ligated vector was pipetted to an ice-cold test tube before 100 µl of competent *E. coli* cells were added using chilled blue tips. After 10 min incubation on ice and 2 min incubation at 37°C the cells were chilled in an ice bath for 2 min. The transformed competent cells were plated on LB-agar plates containing Ampicillin (50 µg/ml). The plates were incubated over night at 37°C.

2.3.5 Heterologous Expression and Purification of Recombinant Protein

2.3.5.1 Induction of the Bacteria Strain ER2566

The cells were grown at 37°C in LB medium containing 100 µg/ml ampicillin. When the OD_{600} of the culture reached 0.5-0.8, protein expression was induced at 15°C over night with IPTG at a final concentration of 0.3 mM. The cells from

the above culture were spun down at 5,000 g for 10 min at 4°C. After discarding the supernatant the cell pellet was stored at -20°C.

2.3.5.2 Preparation of Crude Cell Extract

The cell pellet from a 50 ml culture was resuspended in 5 ml ice-cold cell lysis buffer. The cells were broken by sonication in short pulses of 15 sec for 15 min in ice water. The clarified cell extract was obtained by centrifugation at 12,000 g for 30 min.

2.3.5.3 Purification of the Protein

Intein-tagged protein

Chitin beads were equilibrated at 4°C with 10 volumes of the column buffer prior to the loading of the crude cell extract. The clarified extract was slowly loaded onto the chitin column, which was washed with about 10 bed volumes of the column buffer. The on-column cleavage was conducted by quickly flushing the column with 3 bed volumes of the cleavage buffer containing 50 mM DTT. After a quick flush the flow in the column was stopped, and the column left at 4-23°C for 16-48 h. After the cleavage reaction, the column was eluted with column buffer.

6xHis-tagged protein

One ml of 50 % Ni-NTA slurry was added to 5 ml cleared lysate and mixed gently by shaking at 4°C for 60 min. The lysate-Ni-NTA mixture was loaded into a column with the bottom outlet capped. The column was washed with 4 ml wash buffer. The protein was eluted 4 times with 0.5 ml elution buffer.

Maltose-binding protein-tagged protein

Two ml amylose resin suspension was poured to a column and washed with 16 ml column buffer. 5 ml cleared lysate was loaded onto the column and washed with 24 ml column buffer. The fusion protein was eluted with column buffer containing 10 mM maltose. The elute was dialyzed against 50 mM Tris-HCl (pH 8.0) and treated overnight at RT with enterokinase (20 µg MBP-*Tb*Gpi8 /1 µg enterokinase).

2.3.6 Production of Antibodies Against *Tb*Gpi8

2.3.6.1 Immunization of Chicken with *Tb*Gpi8

The chicken was immunized with isolated MBP-*Tb*Gpi8. After the first immunization with emulsion C the chicken was immunized two times with emulsion I again at the 28th and 42nd day successively.

2.3.6.2 Isolation of the Chicken Antibodies

Three egg yolks were carefully separated from egg whites, mixed with 25 ml phosphate buffer and 75 ml PEG (7 %) and incubated at 4°C for 30 min. After centrifugation at 3000 g for 10 min at 4°C the supernatant was filtered through a mull. PEG was added to the filtrate to a final concentration of 12 % and incubated at 4°C for 30 min. After centrifugation at 3000 g for 10 min at 4°C, the pellet was resuspended in 50 ml phosphate buffer and 50 ml 24 % PEG and incubated at 4°C for 30 min. The mixture was centrifuged at 3000 g for 10 min at 4°C, followed by resuspension of the pellet in 25 ml phosphate buffer and 25 ml ethanol. After centrifugation at 3000 g for 10 min at 4°C the pellet was dissolved in 10 ml phosphate buffer and centrifuged at 3000 g for 10 min at 4°C again. The supernatant was stored at -20°C.

2.3.6.3 Affinity Purification of Chicken Anti-*Tb*Gpi8 Antibodies

10 ml isolated chicken antibodies were subjected to a 1 ml amylose column containing only MBP. The flow through without antibody against MBP was pooled and put onto a second 1 ml amylose column containing the fusion protein MBP-*Tb*Gpi8. The column was washed with phosphate buffer thoroughly. In this course, specific antibody against *Tb*Gpi8 was bound on the column and was eluted with elution buffer (pH 2.5) thereafter.

2.3.6.4 ELISA of Chicken Anti-*Tb*Gpi8 Antibodies

The 96 well micro plate was incubated with 100 ng MBP-*Tb*Gpi8/100 µl coating buffer at 4°C overnight and washed 2 times with 200 µl wash buffer. After incubation with 100 µl blocking buffer at RT for 30 min, it was incubated with 100 µl serially diluted anti-*Tb*Gpi8 antibody at RT for 1 h, followed by a 2 times wash with 200 µl wash buffer. Each well of the micro plate was then incubated

with 100 µl IgY/POD (1:5,000) at RT for 1 h. After 2 times washing 100 µl substrate solution was added and incubated at RT for 30 min in the dark. After addition of 50 µl stop reagent the plate was measured at 492 nm using an ELISA reader.

2.3.7 Characterization of *Tb*Gpi8

2.3.7.1 *In vitro* Test of the Anchor Exchange Activity

Intein-tagged GFP-VSG221-C-terminus and fluorogenic peptide substrates Ac-S-V-L-N-AMC were used as substrate for this reaction. The enzyme sources were trypanosomal lysates (as control), *E. coli* lysates or enterokinase treated MBP-*Tb*Gpi8 or 6xHis-tagged *Tb*Gpi8. The cell lysates or enterokinase treated MBP-*Tb*Gpi8 or 6xHis-tagged *Tb*Gpi8 were mixed with GFP-VSG221-C-Terminus or Ac-S-V-L-N-AMC and 10 mM hydrazine. The mixture was incubated at 30°C for 1 h or overnight and analysed by SDS-PAGE, Western blotting or spectrofluorimeter.

2.3.7.2 Immunolocalization of *Tb*Gpi8

The localization of *Tb*Gpi8 was detected using immunofluoroscopy using affinity purified chicken anti-*Tb*Gpi8 antibodies. FITC conjugated anti-chicken IgG was used as second antibody.

2.3.7.3 Western Blotting Analysis of *Tb*Gpi8 in Trypanosomal Lysates

Cell lysates from bloodstream form cells were separated through SDS/PAGE. Affinity purified chicken anti-*Tb*Gpi8 antibodies were used as first antibody and peroxidase conjugated anti-chicken IgG was used as second antibody.

3 Results

3.1 *In vitro* Translation

3.1.1 Endogenous Translation

Since 1988, cell-free systems from trypanosome bloodstream form have been investigated to link translation and different modification of VSG in our laboratory. By conventional production of cell lysates, a translation inhibitor becomes active, which can only be removed by dialysis, ultracentrifugation with a sucrose gradient or gel filtration. Hence, an *in vitro* translation system from trypanosomes without this inhibitor was developed in our laboratory to study the post-translational modification of VSG.

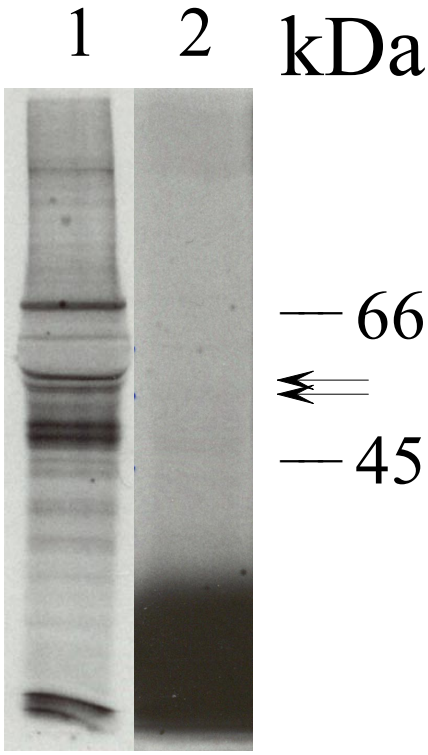

Fig. 3.1: Fluorographies of *in vitro* Translated Proteins from Trypanosomal Lysates

In vitro translation assays were performed in trypanosomal lysates, which were not treated (lane1) or treated (lane 2) with Ca^{2+}-dependent nuclease S7. The nuclease S7 treated trypanosomal lysate was supplemented with isolated trypanosomal RNA at 0.2 µg/µl. Newly formed proteins were labeled with [^{35}S]-methionine. VSG bands are represented by arrows.

To avoid liberation of lysosomal constituents, a 'limited lysis' was applied to the preparation of translational active lysates. Trypanosomes were hypotonically lysed in double-distilled water containing DTT, 2-aminopurine and proteinase

inhibitors. The cells were homogenized for 7 min using a tight fitting potter. The tonicity was adjusted to nearly isotonic conditions by adding 10 % (v/v) of phosphate buffer before the sample was centrifuged (6 min, 12000 g, 4°C) to obtain the post-mitochondrial supernatant (PMS). This lysate was very efficient for *in vitro* translation after supplemented with an oxidation protector (DTT), energy resources (ATP, GTP and ATP regenerating system), different ions and amino acids. An incorporation of about 50,000 dpm/µl lysate was regularly obtained. As shown in Fig. 3.1, VSG was the primary translation product. Radiolabeled protein bands ranged from 7.3 kDa to 200 kDa (Hömke, 1997). However, upon digestion of the lysate with Ca^{2+}-dependent nuclease S7 and addition of total RNA from trypanosomes, incorporation of radio labeled amino acid was very poor, although different RNAs and different techniques to remove endogenous mRNAs were tried and tested. Newly formed proteins could not be detected by SDS/PAGE/fluorography (lane 2). Thus, reinitiation does not occur in our *in vitro* translation system. Therefore all the following work was carried out with this system using endogenous mRNA.

3.1.2 *In vitro* Translation Using Reticulocyte Lysates

The reticulocyte lysate system is the most widely used eukaryotic cell-free protein synthesizing system because of the high translational activity with endogenous or exogenous mRNAs. It is easier to study *in vitro* modification, if the produced protein is different to endogenous proteins from the used cell-free system in immunological and chemical characters.

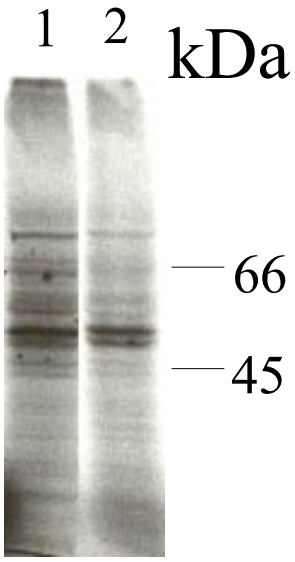

Fig. 3.2: Fluorographies of *in vitro* Translated Proteins from Reticulocyte Lysates

In vitro translation assay was performed in reticulocyte lysates (lane 1) or in reticulocyte lysates supplemented with trypanosomal lysates (lane 2) that was treated with Ca^{2+}-dependent nuclease S7. Newly formed proteins were labeled with [^{35}S]-methionine and visualized by fluorography.

A commercially available reticulocyte lysate was used to reinitiate the translation of mRNA from trypanosomes and to test the modification ability of the supplemented trypanosomal lysate. Trypanosomal lysates were treated with nuclease S7 in the presence of 1 mM Ca^{2+} for 20 min at room temperature before EGTA was used to inactivate the nuclease. The lysate was then mixed with reticulocyte lysate. As shown in Fig. 3.2, this mixture did not lead to any change of newly formed proteins. Thus post-translational modification could not be induced in reticulocyte lysate by supplementing trypanosomal microsomal membranes.

3.1.3 N-Glycosylation of *in vitro* Translated VSG

To show N-glycosylation of *in vitro* translation products in our cell-free system, the lysate was subjected to N-glycosidase F digestion after translation. This enzyme cleaves all types of Asn-linked N-glysidic linkage between asparagine and N-acetylglycosamine residues.

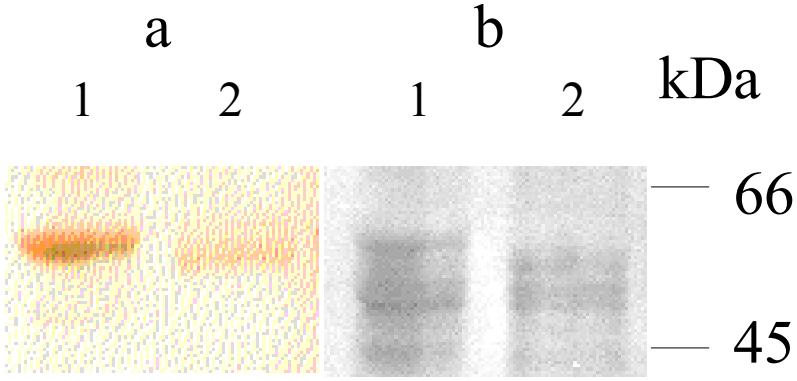

Fig. 3.3: Glycosylation of *in vitro* Translated VSG

Lysates of clone MITat 1.4 were incubated for 1 h at 30°C. Newly formed proteins were labeled with [^{35}S]-methionine. After *in vitro* translation, VSG was dissolved with *n*-octylglucopyranoside and treated with N-glycosidase F as described in materials and methods. After SDS/PAGE, newly formed proteins were visualized by fluorography (panel b); deglycosylation of VSGs was controlled by concanavalin A blotting (panel a). Lane 1 is the sample not treated with N-Glycosidase F; lane 2 is the sample treated with N-glycosidase F.

As shown in Fig. 3.3b, the newly formed VSG band disappeared and resulted in a new band with lower apparent molecular mass, indicating that newly synthesized VSG underwent translocation and glycosylation in the cell-free system. Concanavalin A is a kind of lectins from Jack Bean, which shows high affinity for terminal α-D-mannosyl and α-D-glucosyl residues. Ca^{2+} and Mn^{2+} ions are required for activity. In this experiment, the lysate of trypanosome bloodstream form MITat 1.4 was used, which expresses a VSG of the oligomannose-type N-glycan with 3 terminal α-D-mannosyl residues. To further test the efficiency of the digestion, concanavalin A was used on the blotting (Fig 3.3a). All VSGs were efficiently digested to a new band with lower molecular weight and with a relative weak staining. This weak staining was due to the mannose residues of the core structure of the GPI anchor. Because the 'old' VSG runs together with the newly formed VSG, this experiment could not tell if the newly formed protein was attached with GPI anchor or not, while N-glycosylation could be clearly demonstrated.

3.1.4 Existence of Pro-VSG

Normally the C-terminus of VSG contains 17~23 amino acids, which is absent in mature VSG. This C-terminus is very hydrophobic and probably used as a temporary membrane anchor (Duszenko & Seyfang, 1993). About one minute after completion of the translation, the pro-VSG is processed to mfVSG by replacing the C-terminus with the preformed GPI anchor, which forms an amide bond with the C-terminal amino acid of VSG (Bangs *et al*., 1985; Ferguson *et al*., 1986). After *in vitro* translation, the samples were detected using antibodies against the VSG C-terminus to check for pro-VSG. Fig. 3.4 shows the results of Western blotting and corresponding fluorography. In lane 1, a protein band with C-terminus is located at about 55 kDa and in lane 2 a corresponding band visualized by fluorography is also located at the same position. Therefore, this experiment suggests that some pro-VSGs are not changed into mf-VSG immediately during the VSG processing course. There was always some VSG remained as pro-VSG. In addition, the cell sample not undergoing *in vitro* translation showed also a pro-VSG band (data not show). These results indicate that this may be a normal phenomenon *in vivo*.

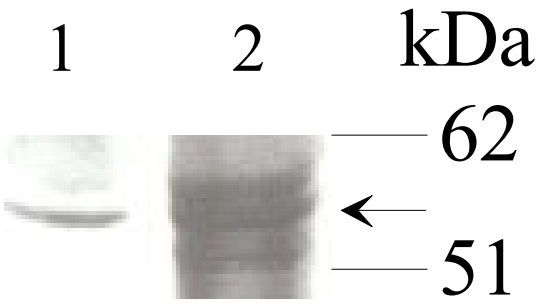

Fig. 3.4: Western Blotting and Fluorography Analysis of the Pro-VSG

An *in vitro* translation assay was performed with lysate MITat 1.2. Newly formed proteins were labeled with [^{35}S]-methionine. After SDS/PAGE, proteins were blotted onto a nitrocellulose membrane. The pro-VSG was detected with anti-C-terminus antibody (lane 2); newly formed proteins were visualized by fluorography (lane 2).

3.1.5 GPI Anchoring of the VSG Formed during *in vitro* Translation

3.1.5.1 The Biotin-Lysine System

In vitro translation assays have traditionally been monitored by following the incorporation of a radiolabeled amino acid into newly synthesized protein. There is an alternative nonradioactive biotin-labeling method. $tRNA^{Lys}$ is first aminoacylated with lysine, which is then chemically labeled with biotin, modified in the ε-position of the amino acid. If biotin-lysine-$tRNA^{Lys}$ is added to the *in vitro* translation system, the biotinylated lysine is incorporated into the growing polypeptide chain. Since preexistent proteins always interfere with the modification analysis of the newly formed proteins, the biotin-lysine was used in an *in vitro* translation system instead of [^{35}S]-methionine to label newly formed proteins to solve this problem. Using this method, newly formed proteins could be isolated from the lysate by affinity chromatography using avidin-linked agarose. In following tests, Biotin-lysine was efficiently incorporated to newly formed proteins. These proteins could be detected by blotting the SDS/PAGE gel onto a nitrocellulose membrane and visualized using a strepavidin detetion kit. The protein distribution detected by fluorography was similar to those detected by streptavidin (Fig. 3.5). Again, VSG was the primary translation product.

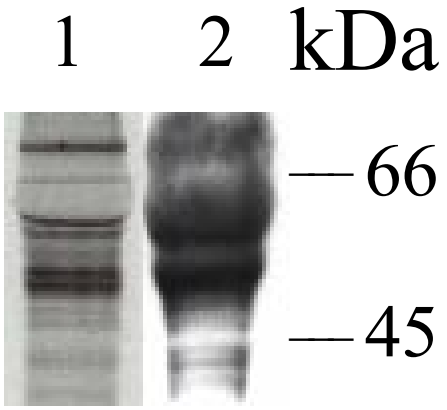

Fig. 3.5: Comparison of the *in vitro* Translation Using [^{35}S]-Methionine and Biotin-Lysine-tRNA^{Lys}

In vitro translation was performed using MITat 1.4 lysate. The newly formed proteins were labelled with [^{35}S]-methionine (lane 1) or biotin-lysine (lane 2) respectively in the presence of 7.5 kBq/µl [^{35}S]-methionine or 0.25 pmol/µl biotin-lysine-tRNA^{Lys}.

3.1.5.2 Detection of Membrane Anchor Exchange Using Anti-CRD-Antibodies

The presence of a biotin tag on *in vitro* translated proteins enables the selective capture of biotinylated translation products using immobilized streptavidin. Neither translocation across the endoplasmic reticulum nor glycosylation is seriously affected by incorporation of the biotin moiety into the protein chain (Kurzchalia *et al*., 1988). It became possible to specifically isolate 'new' VSG and to detect it on Western blot with specific antibodies. In the cell lysate, the highly efficient endogenous GPI-PLC should convert mfVSG to sVSG. This enzyme cleaves the phosphodiester bond between phosphate and diacylglycerol with the formation of a 1,2-cyclic phosphate ring on the inositol residue, which is the primary epitope for anti-CRD antibodies. This cross-reaction is one of the main criteria used in the identification of a GPI anchor (Zamze *et al*., 1988). For characterization of the anchor attachment in the newly formed VSG, anti-CRD-antibodies were used in the following experiments. Due to the presence or absence of the cyclic phosphate residue, anti-CRD-antibody specifically reacts with the anchor of sVSG, whereas it does not reacted with mfVSG. In our *in vitro* translation system the mfVSG could be rapidly transformed to sVSG with high efficiency. The newly formed VSG could also be transformed to sVSG immediately after appearance. After the translation mixture was incubated at 30°C for 1h, it was subject to affinity precipitation using immobilized avidin agarose. After SDS/PAGE and blotting, the blot was probed with CRD antibodies, VSG antibodies and peroxidase linked streptavdin respectively. As

shown in Fig. 3.6, an avidin-agarose precipitated protein (lane 1) could be detected not only with streptavidin and MITat 1.4 VSG-antibodies but also with CRD-antibodies, indicating that this protein band is newly formed VSG in our *in vitro* translation system and it is also attached with GPI anchor. The CRD antibodies do not react with mfVSG (Panel 2, lane 3), suggesting that the antibody is very specific for the GPI anchor in sVSG with CRD epitope, and shows no cross reactivity with VSG. These results clearly demonstrate the *in vitro* GPI anchor exchange in our cell-free system.

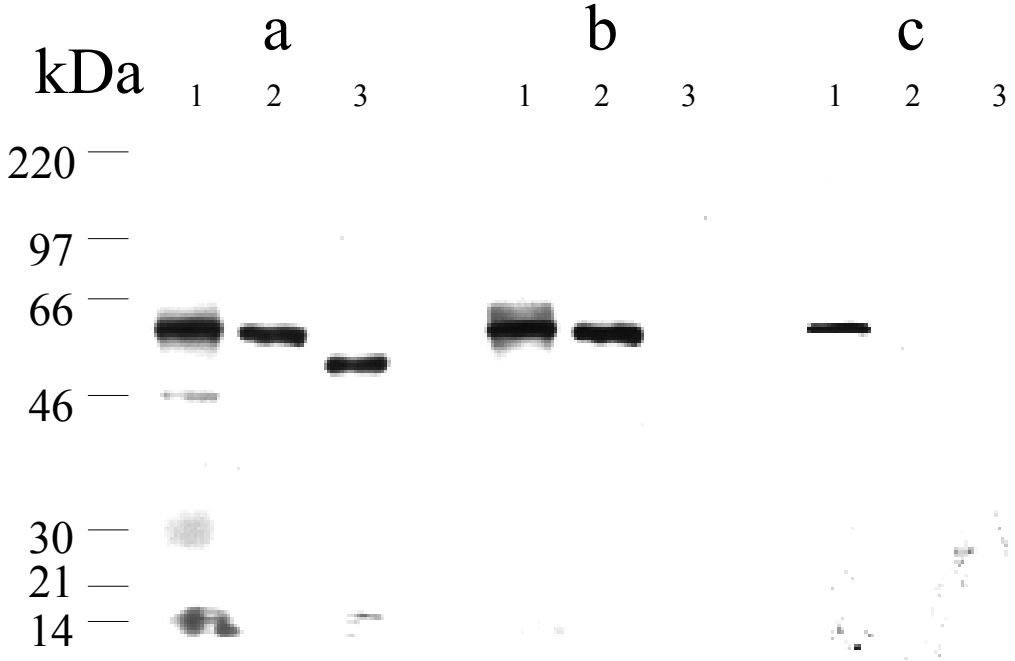

Fig. 3.6: GPI Anchor Additon *in vitro*

During *in vitro* translation, proteins were labeled with biotinylated lysine and then precipitated using immobilized avidin. After SDS/PAGE, proteins were transferred onto a nitrocellulose membrane and were detected with clone-specific antibodies (MITat 1.4) for VSG (panel a), CRD-specific antibodies for GPI anchor (panel b) and streptavidin-horseradish peroxidase (panel c) for newly formed VSG. Lane 1 shown the proteins that affinity precipitated using avidin-agarose; lane 2 and lane 3 are sVSG and mfVSG used to control the specificity of anti-CRD antibodies.

3.1.5.3 $ZnCl_2$ Inhibition of GPI-PLC

During VSG synthesis, mfVSG should be the first product, which is then transformed into sVSG. To identify this process, $ZnCl_2$, a very efficient inhibitor of GPI-PLC, was used in *in vitro* translation. In this experiment the GPI-PLC activity was successfully inhibited by addition of 0.2 mM $ZnCl_2$ to obtain mfVSG. $ZnCl_2$ at this concentration reduced translation efficiency only marginally. Following *in vitro* translation, samples were centrifuged (100,000 g, 1 h, 4°C) to separate membrane-bound proteins and soluble proteins. After SDS/PAGE and Western blotting, newly formed proteins were detected by streptavidin-horseradish peroxidase. As shown in Fig. 3.7, in the absence of $ZnCl_2$ (panel a), all VSG was found in the supernatant fraction. Due to the endogenous GPI-PLC, mfVSG was readily transformed to sVSG. In the presence of $ZnCl_2$ (panel b), about 50% VSG existed in the pellet fraction as mfVSG. However, although the amount of mfVSG clearly increased compared with the control in the absence of $ZnCl_2$, a total inhibition of the endogenous GPI-PLC was not achieved, because 0.2 mM $ZnCl_2$ represents the IC_{50} for this enzyme (Cross, 1984).

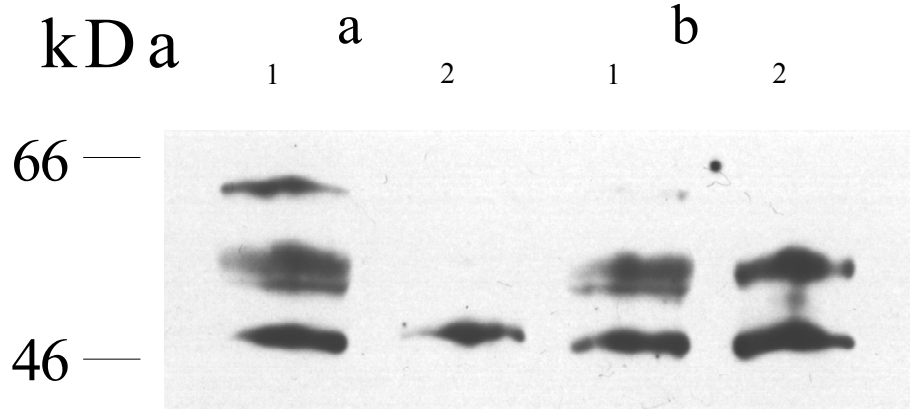

Fig. 3.7: Effect of Zinc Chloride on GPI-PLC

In vitro translation assay was performed in the presence (panel b) or absence (panel a) of $ZnCl_2$. Following *in vitro* translation, lysates were centrifuged (100,000 g, 1h, 4°C) to separate supernatant (lane 1) and pellet (lane 2). After SDS/PAGE, proteins were blotted onto a nitrocellulose membrane. Biotinylated proteins have been detected using streptavidin-horseradish peroxidase.

P-chloromercurybenzen-sulfonic acid, other GPI-PLC inhibitor, and higher concentration of $ZnCl_2$ inhibited translation distinctly. They could not be used for this purpose. Inhibition of the GPI-PLC provided additional evidence for GPI anchor exchange in our cell free system.

3.1.5.4 Galactosylation of GPI Anchor in VSG of Clone MITat 1.4

The core structure of GPI anchor is identical in all VSG variants, while the galactose side branch varies. VSG of clone MITat 1.4 contains a galactosylated GPI anchor, but not a galactosylated oligomannose type N-glycan. After *in vitro* translation, proteins were separated by SDS/PAGE and transferred onto a Western blot. The results show a protein band, which was detected with anti-VSG antibody (Fig. 3.8c). This band was also labelled by biotinylated lysine (Fig. 3.8a) and UDP-[^{14}C]galactose (Fig 8b). Since in this clone, galactosylation occurs only on the GPI-anchor, the labelling of UDP-[^{14}C]galactose on VSG clearly suggests that the *in vitro* translation system is able to produce GPI anchored galactosylated protein. Unlike the L-[^{35}S]-methionine, UDP-[^{14}C]galactose induced relative poor incorporation of the radioactivity. This is might be due to a chase by the existence of endogenous galactose.

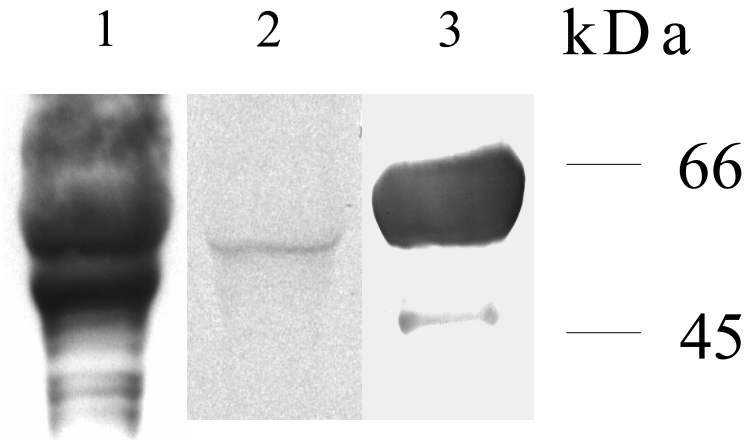

Fig. 3.8: Double Labelling with Biotinylated Lysine and UDP-[^{14}C]Galactose

Newly formed proteins were labeled *in vitro*, separated on SDS/PAGE and transferred onto a Western blot. The newly formed proteins were double labeled using biotinylated lysine and UDP-[^{14}C]-galactose. After SDS/PAGE and Western blotting, the blot was analyzed by using anti-VSG antibodies (lane 3), streptavidin-horseradish peroxidase (lane 1) and fluorography (lane 2).

3.2 Investigation of the Anchor Exchange Mechanism *in vivo*

3.2.1 Cell Cultivation in the Presence of Hydrazine

The proposed mechanism of GPI transfer is a transamidation reaction that involves the formation of an activated carbonyl intermediate (enzyme-substrate complex) with the ethanolamine moiety of the preassembled GPI unit serving as a nucleophile. Hydrazine and hydroxylamine are well known to be nucleophilic acceptors in the transpeptidase (Tate *et al*., 1974) and transamidase (Buchanan *et al*., 1973) reactions. They have also been shown to serve as possible alternative substrates for GPI using engineered protein miniplacental alkaline phosphatase (prominiPLAP) and rough microsomal membranes of HeLa cells. The use of readily available nucleophilic substitutes such as Hydrazine and hydroxylamine is a viable alternative to study COOH-terminal processing by the putative transamidase (Maxwell *et al*., 1995; Ramalingam *et al*., 1996). For confirmation of the same transamidase mechanism in trypanosomes, hydrazine was first used in culture media to determine its toxicity, before it was used to investigate its effect on anchor transfer at an appropriate concentration. According to the initial experiment, trypanosomes could live in 10 mM hydrazine, the normally used concentration in the *in vitro* anchoring assay, for about 5~6 h, but stayed alive for more than 44 h in 0.5 mM hydrazine.

3.2.2 Nonglycosylated VSG and Tunicamycin

Tunicamycin inhibits an early step in the formation of asparagine-linked oligo-saccharides. It is a hydrophobic analogue of the UDP-N-acetylglucosamine andblocks the addition of N-acetylglucosamine to dolichophophate, the first step to form the core-oligosaccharide. In order to produce a VSG showing a different molecular weight as the old VSG, cells were cultivated in the presence of 10 µg/ml tunicamycin. After 4 h in culture, two VSG bands with a distinctively lower molecular weight were successfully produced and could be detected on the Western blots with anti-VSG antibodies (Fig. 3.9), glycosylation of VSG was efficiently inhibited by tunicamycin. These VSGs are allowed to analysed after SDS/PAGE separation using different methods without interfere of the normal VSG.

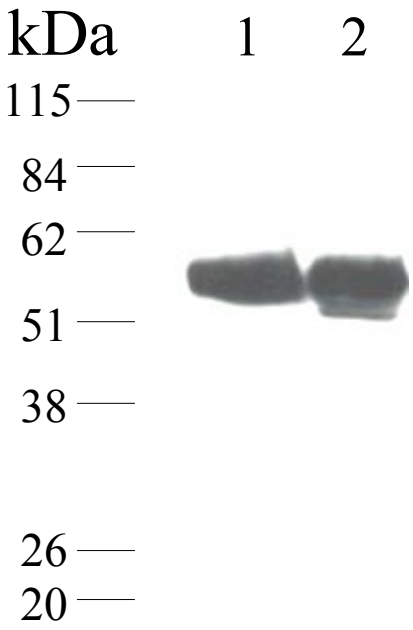

Fig. 3.9: Western Blotting Analysis of the *in vivo* Synthesized VSGs Using Anti-VSG MITat 1.2 Antibodies

The cells were cultured in the presence (lane 2) and absence (lane 2) of 10 µg/ml tunicamycin. After 4 h incubation at 37°C and SDS/PAGE, the proteins were transferred onto a nitrocellulose membrane and detected by using anti-VSG MITat 1.2 antibody.

3.2.3 Hydrazine and Hydrazine-Biotin as Substitutes for GPI in Transamidation Reaction

Previously, a putative mechanism for the transamidase-mediated nucleophile incorporation at the C terminus of protein was proposed (Udenfriend *et al*., 1995; Sharma *et al*., 1999). The carbonyl group of the ω amino acid of pro-VSG is activated by a sulfhydryl group in the transamidase resulting in the formation of an enzyme-substrate complex and cleavage of the amide bond between ω and ω + 1 of pro-VSG. Nucleophilic attack by H_2N-X results in formation of VSG-NH-X and regeneration of the active site sulfhydryl in the transamidase. If hydrazine or hydrazine-biotin were used as substitutes for GPI, VSG-NH-NH_2 or VSG-NH-NH-biotin would be produced without GPI anchor and thus could not be detected by anti-CRD antibodies. To demonstrate this transamidase mechanism, trypanosomes were cultivated in the presence of 10 mM hydrazine or 10 mM hydrazine-biotin for 4 h. After SDS/PAGE and Western blotting, the same blot was analysed using anti-MITat 1.2 antibodies (Fig. 3.10a), anti-CRD-antibodies (Fig. 3.10b) and streptavidin-HRP (Fig. 3.10c). If trypanosomes were cultivated in the absence of hydrazine and hydrazine-biotin, the produced VSG band could be labelled using anti-CRD antibodies (Fig. 3.10, panel b2) while cells cultured in the presence of hydrazine or hydrazine-biotin could not be detected (Fig. 3.10, panel b3 and b4). This result suggests that the GPI was

successfully replaced by hydrazine and hydrazine-biotin. To further prove this finding, the blot was additionally tested with streptavidin. Fig. 3.10c shows that cells cultured in presence of hydrazine-biotin produced biotin labelled VSG. These results show that hydrazine could enter the parasite, function as a nucleophile in the transamidation reaction and form a hydrazide derivative of VSG. All of these results coincide with the putative anchor exchange mechanism.

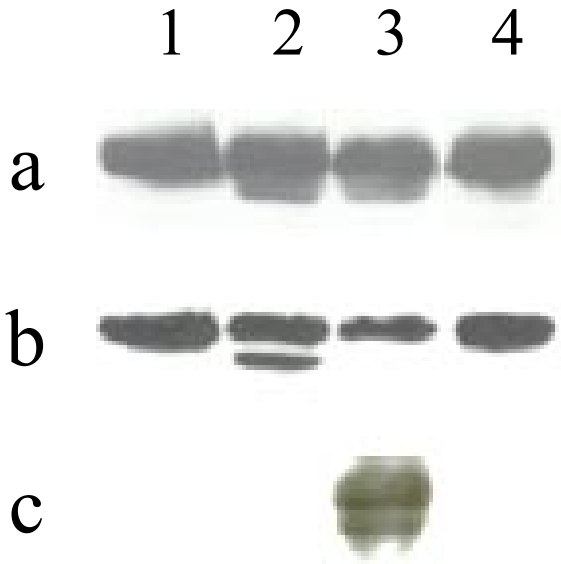

Fig. 3.10: Effects of the Hydrazine and Hydrazine-Biotin in the Anchor Exchange Reaction

Trypanosomes were cultivated in the presence of 10 µg/ml tunicamycin (lane 2, 3 and 4) and 10 mM hydrazine (lane 3) or hydrazine-biotin (lane 4) for 4 h at 37°C. The control samples were incubated in the absence of tunicamycin (lane 1) or hydrazine (lane 2). After cultivation, the cells were subjected to SDS/PAGE and analysed by western blotting. Anti-VSG MITat 1.2 antibody and anti-CRD antibody were used to detect VSG (panel a) and GPI anchor (panel b). Streptavdin was used to detect the biotin tag on VSG (panel c).

3.2.4 Immunofluoroscope of the Hydrazine Treated Trypanosomes

Since in cultivated cells hydrazine was incorporated into VSG, this would result in the release of a water-soluble derivative of VSG from cell membrane. In trypanosomes, VSG intracellular transport and export are critically dependent on

the presence of GPI anchor (Bangs *et al*., 1996 & 1997). This is a common feature of GPI-anchored proteins in trypanosomatid protozoa. McDowell *et al* (1998) have investigated the role of GPI-anchor in forward secretory trafficking using African trypanosomes. Soluble GPI-minus forms of VSG, in which the C-terminal GPI-addition peptide signal is deleted, are secreted with a 5-fold reduced kinetic. Immunofluorescence localization studies indicate that the GPI-minus VSG accumulates in the endoplasmic reticulum (ER). Since VSG-hydrazide is a kind of GPI-minus VSG and should have a similar secretory retardation and/or accumulation.

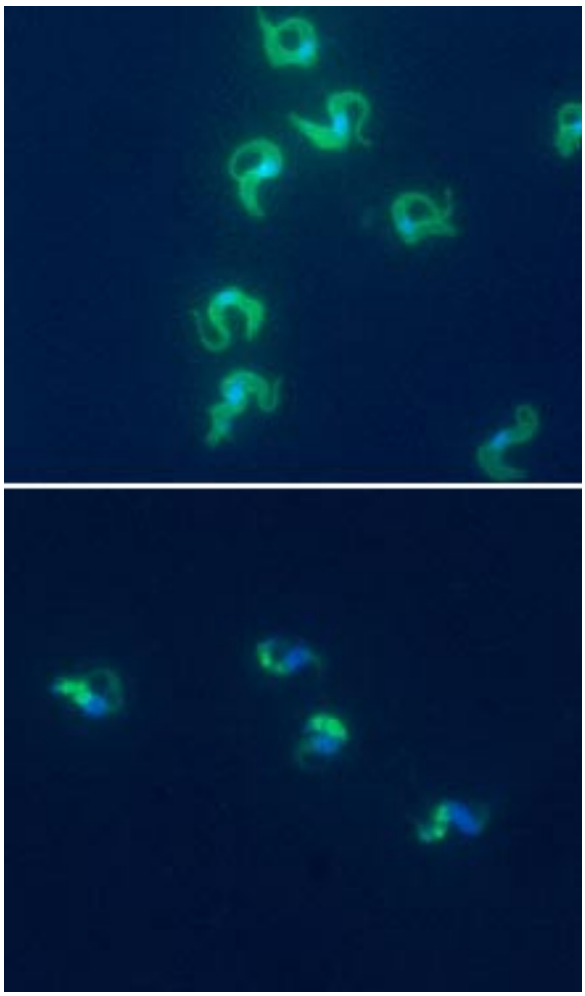

Fig. 3.11: Immunolocalization of VSGs before and after Treatment with Hydrazine

Cells were cultured in the presence (panel b) or absence of hydrazine (panel a). After 44 h of incubation at 37°C, the parasites were fixed in HBS buffer containing 1% formaldehyde. VSG was localized using anti-VSG MITat 1.2 antibodies.

To determine the location of VSG-hydrazid, immunofluorescence assays were performed using cultivated bloodstream form MITat 1.2 cell line. Fixed cells were stained with antibodies against MITat 1.2 VSG. The cell nucleus and

kinetoplast were stained with bisbenzimid. As shown in Fig. 3.11, VSG was equally distributed on the cell surface and stained no obvious visible vesicle within the cell, if the cells were not treated with hydrazine (panel a). In contrast, in the hydrazine treated cells (panel b) there were a few intensive stained vesicles, which located between cell nucleus and kinetoplast. On the cell surface, relatively little fluorescence was produced. These results are readily explained that hydrazine as a substitute of GPI incorporated into VSG resulting in produce of soluble GPI-minus VSG --- VSG hydrazid. VSG hydrazid could not be transported to the cell surface leading to accumulation of them within cell organelles. This accumulation is consistent with the Western blotting results and verified the transamidase reaction mechanism by GPI anchor exchange.

3.3 Measurements of Transamidase Activity in Trypanosomal Lysate Using Fluorogenic Peptide Substrates

3.3.1 Fluorogenic Peptide Substrates and Enzyme Assay Conditions

The use of 7-amino-4-methylcoumarin (AMC) fluorogenic peptide substrates is a well-established method for the determination of protease specificity (Zimmerman *et al*., 1977). The substrate is extremely stable in solution and closely related to the natural peptide substrates. Specific cleavage of the anilide bond liberates the fluorogenic AMC group, thus allowing the simple determination of cleavage rates for individual proteases. The cleavage site for the GPI attachment (ω-site) in VSG is always one of the following three amino acids: Asp, Ser or Asn. In this assay Ac-S-V-L-N-AMC was used as a substrate to test the transamidase activity in lysates. The excitation and emission maxima of the amino-conjugated AMC substrates are 350 nm and 400 nm, respectively. Cleavage of the substrate by the transamidase to release the free AMC results in a shift of the excitation and emission maxima to 340 nm and 440 nm, respectively. Hydrolysis of AMC substrates was monitored fluorimetrically with an excitation wavelength of 360 nm and an emission scan from wavelength of 370 nm to 530 nm on a spectrofluorimeter. The substrate was incubated together with trypanosomal lysates at 30°C for 1 h using 1 mM substrate in 50 mM citrate buffer at pH 6.5. As shown in Fig. 3.12, the emission maxima changed from 400 nm to about 440 nm after addition of trypanosomal lysates, suggesting

that the lysates contained an active transamidase, which catalysed the cleavage of the anilide bond.

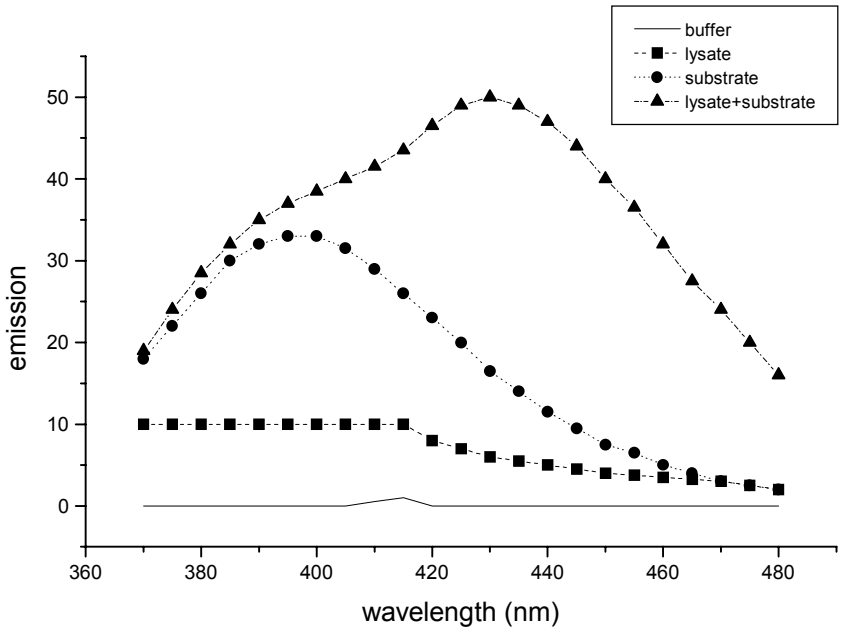

Fig. 3.12 : Transamidase Activity in the Trypanosomal Lysate

1 mM substrate was incubated with lysate at 30°C for 1 h. After incubation, the shift of emission maxima in samples was measured. Lysate and buffer were also measured at mock condition.

3.3.2 Optimum pH of the Transamidase

The optimum pH of the transamidase was detected by using trypanosomal lysate and Ac-S-V-L-N-AMC. Release of the fluorogenic AMC was dependent on pH value. The highest emission was acquired at pH 5.5, as can be seen from Fig. 3.13. A rapid decrease in activity was measured at more acidic and basic condition. At pH 4.5 and pH 6.5 only about 10% activities were left. This result is consistent with the molecular feature of cloned *Tb*Gpi8, which has a pI of 5.51. It shows a similarity with legumain, an asparaginyl endo-peptidase with a pH optimum 5.8 (Chen *et al*., 1997).

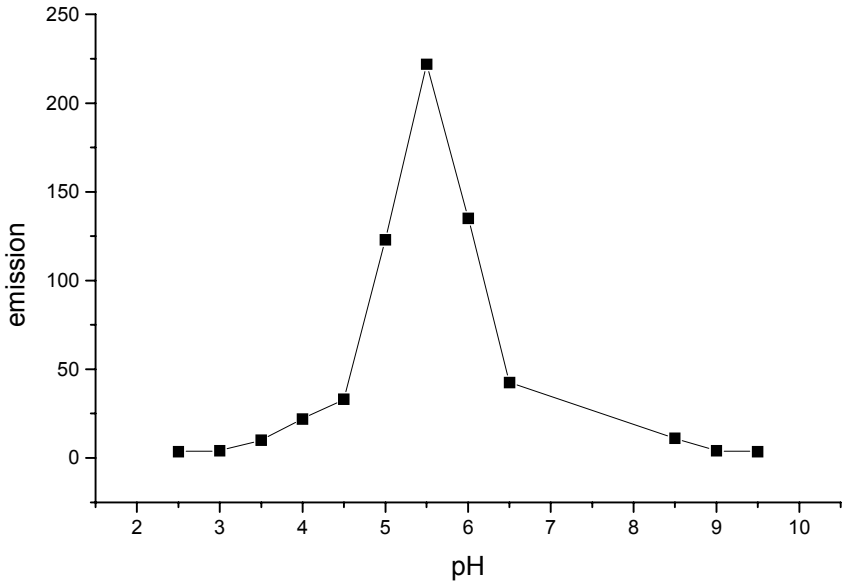

Fig. 3.13: Optimum pH of the Transamidase in Lysate

1 mM substrate was incubated with 10% trypanosomal cell lysate at 30°C overnight in 50 mM citrate buffer ranging from pH 2.5 to 6.5 and 50 mM Hepes buffer ranging from pH 5.5 to 9.5. The emission at 440 nm was measured on a spectrofluorimeter as described.

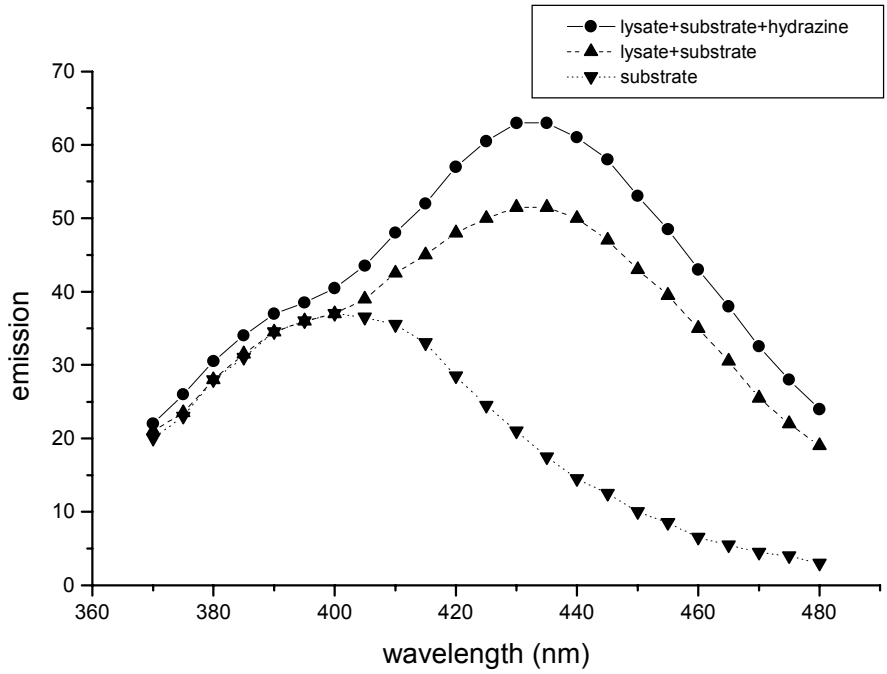

Fig. 3.14: The Effect of Hydrazine on Transamidase

Cell lysates were incubated with 1 mM substrate at 30°C for 1 h in the presence or absence of 10 mM hydrazine. After incubation, the shift of emission maxima in samples was measured in a range from 370 nm to 480 nm.

3.3.3 The Effect of Hydrazine on Transamidase Activity

Nucleophilic attack by GPI, water or a nucleophile such as hydrazine is the final step of the transamidation reaction. In the reaction mixture, this reaction could be stimulated following the addition of 10 mM hydrazine. At about 440 nm a higher emission was observed than the sample without hydrazine (Fig. 3.14).

3.3.4 DDT, pCMPSA and Transamidase Activity

As previously shown, the GPI anchoring reaction in bloodstream stage trypanosomes is inhibited by the sulfhydryl alkylating reagent *p*-chloro-mercuriphenyl sulfonic acid (pCMPSA) (Mayor *et al*., 1991; Sharma *et al*., 1999) and it was thus supposed that a His and/or a Cys residue is located at the active site (Meyer *et al*., 2000). The effect of pCMPSA was thus detected in our assay.

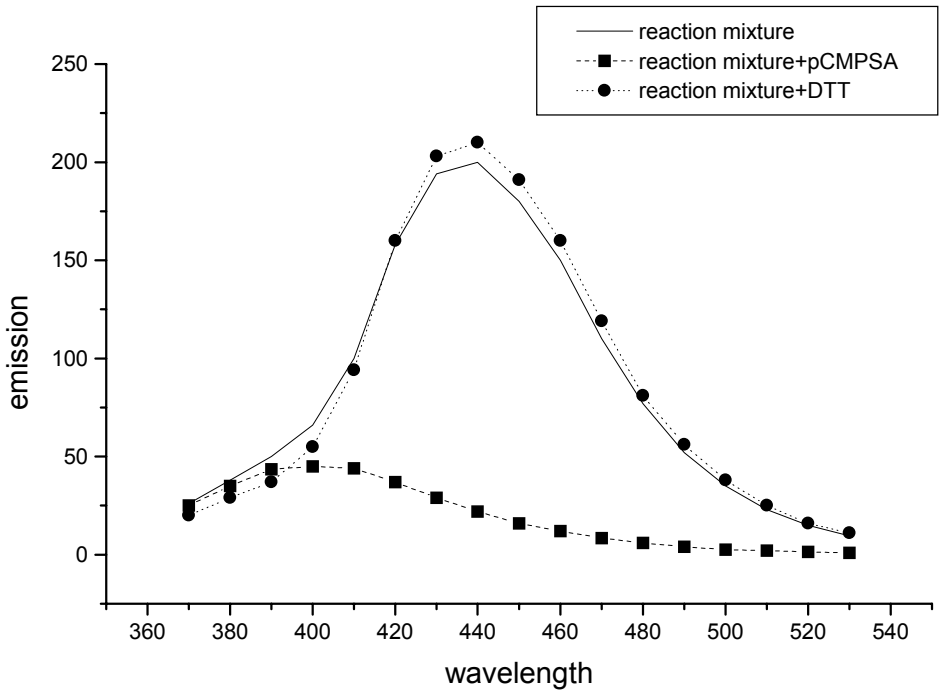

Fig. 3.15: Inhibition of Transamidase with Sulfhydryl Alkylating Reagent

Cell lysates (10%) were incubated with 1mM substrate in the presence of 1 mM pCMPSA or 1 mM DTT at 30°C overnight.

As shown in Fig. 3.15, the release of AMC from fluorogenic peptide substrate was clearly inhibited. This result is consistent with the assumption that the trypanosome GPI transamidase contains a catalytically important sulfhydryl residue. The effect of DTT was also detected using the substrate. However, no obvious stimulation was acquired. It is possible that in the cell lysate existed enough amount of DTT for the enzyme activity, which was supplemented during lysate preparation.

3.4 *Tb*Gpi8

3.4.1 Cloning of *TbGPI8*

Degenerated oligonucleotide primers were designed against highly conserved regions based on those of yeast and human by using BLAST programme (http://www.ncbi.nlm.nih.gov/) and DNA fragments of T. brucei sequenced by TIGR (TIGR –Databases, http://www.tigr.org/). The 5'-sequence of the *TbGPI8* was amplified using primers GPI8AS1 and SLSE23 and cloned into pBS KS^+. The 3'-sequence was amplified using primers GPI8SE3 and OT203N and cloned into pBS KS^+. cDNA from the bloodstream form MITat 1.2 was used as template. After sequencing by GATC, 2 specific primers GPI8SE1 and GPI8AS2 were used to amplify the *Tb*Gpi8-ORF from bloodstream form MIT1.2 cDNA and cloned into pBS KS^+. 2 clones were sequenced from the T7 and T3 promoters of pBS KS^+. Both clones gave the same sequence results. They are 1533 bp in length. The ATG start codon is at position 179. The ORF is 960 bp long including the stop codon TAG and codes for a polypeptide of 319 amino acids. The cDNA sequence and the corresponding protein sequence are given as follows. The start and stop codons and one N-glycosylation site and signal sequence are shown in bold face and underlined:

TbGPI8:

```
1       CGCTATTATT AGAACAGTTT CTGTACTATA TTGAAAAGTG TTAGATAGTT AAGTTAAATA
61      AGAAATTTAA GTTGTAGTCC CCATCGCCGA GTACTGGGTT CGCACCAGCA CGGACGAGGA
121     TAGGCGCAGA GGTTTCAAAC AAGTGGCAAG GGAAGAGTTG GTAAAGGGTT GCATTAACCT
181     GAAGTGTGCT GACGTGGAAG GAGAAACCGA CGGCACTTAG GTTGAGTACA TAGTATCCGA
241     TTAGTTGGAT CAGGCGCTTG CATATTTATT TCCAGCATTC GTTTTCTTGC AACTGGAAGG
301     TGATGTTGCC CATGTTACTG TGGCTCGTGG CCAACCTCTT CTTAGCGCCG GCCGCGGAAG
361     GCTTTCATGG TATGAATAAA ACCAACACCT GGGCCGTCAT CCTGTCCTCC TCCCGTTACT
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421     TCTTTAATCT CCGTCATACA ACGAATGCGC TGGCCATGTA CCATTTGTGT CGAAAACACG
481     GGATGGATGA CGACCACATC CTTGTCTTTC TTAGCGATAG CTACGCCTGC GATCCCCGCA
541     AACCCAACCC AGCGACAATT TACGGAGCAC CTGCCCAAGC GGAGCAACCG AATCTATACG
601     GCTGCAATAT TAGAGTGGAC TACGCGAGCT ACGATGTCGG TGTTCGCCGG TTCCTTGGCG
661     TCCTGCAGGG CCGATATGAT GAGAATACTC CTCCTTCACG GCGTCTCGAC ACCGACGAGA
721     ACTCGAACAT AATCATTTAC GCTGCTGGCC ACTCAGCTGA GAAATTCTTC AAGTTTCAGG
781     ACTCGGAGTT CATGAGCTCA ACAGACATTG CGGACACCCT CATGATGATG TGGGAACAGC
841     GACGATATCG TAAACTAGTT TTCCTTGTTG ACACATGCCG TGCGCTTTCC TTGTGTCTTG
901     AGATAAAAGC ACCAAATGTC GTTTGCCTCG CCTCTTCCGA AGCGCATTTG GATAGCTACT
961     CGCACCACCT TGACCCTCCA TCAGGCTTCA CTGTCATCAC GCGCTGGACG TTTGAGTTCC
1021    TTGAGGTGCT CAAGGACTCG AAATGTCGGC CAGAAAACGG GGAAGTAACG CTGCTCCAGA
1081    AATCATTTTA TGACTTCAAT TACGGTCCAG AGAGACTGAG TTTGCCCCAA CCACTCTCTG
1141    AACCCGCGCA CTTCGATGCC GTGAACCGTC CAAACGCAAT CCGTGAGTGG AAAATGGACG
1201    AATTTTTCTG TGAGCAGGAT CGGGATAAGA TCCCAGTAGA GTTACGTTAC GATTTGTTCT
1261    AGTTCCACCA AAACCGAGGG GGGTATGTCC CATCAGTTGG AAGGAGAAAA ACGAACTTCC
1321    TTCCCGAAAC TGACTCTCGG TGTCTATCTG GGATGGAGGA GAGAGAAAGA GGAGGGGTGA
1381    AAGTAACATG AGGGGAACAT ATTACTGCTG TGCGATGACA GCACGACAAT ATTTGCTGCC
1441    AATTATGCGG CTGTGTCTGT GCCTGTTATG TGGCTGTCCT TCTCCGTTTG TACATATGAA
1501    ATATTTCTCA AATTTGCGTT CCTTTCTTCG GGGCGGCACC CCACCTCCTT CTGTTATTCC
1561    TACCGGCTTA CGCGGGGCGG CTGTCCGGCA TCGTTAAACC GTCGGGGCAG GACGAAAAAA
1621    AATAATTATA ACAATATTGT AGTCACGTGC AACAAAGCCG
```

*Tb*Gpi8p :

```
1       MLPMLLWLVA NLFLAPAAEG FHGMNKTNTW AVILSSSRYF FNLRHTTNAL AMYHLCRKHG
61      MDDDHILVFL SDSYACDPRK PNPATIYGAP AQAEQPNLYG CNIRVDYASY DVGVRRFLGV
121     LQGRYDENTP PSRRLDTDEN SNIIIYAAGH SAEKFFKFQD SEFMSSTDIA DTLMMMWEQR
181     RYRKLVFLVD TCRALSLCLE IKAPNVVCLA SSEAHLDSYS HHLDPPSGFT VITRWTFEFL
241     EVLKDSKCRP ENGEVTLLQK SFYDFNYGPE RLSLPQPLSE PAHFDAVNRP NAIREWKMDE
301     FFCEQDRDKI PVELRYDLF
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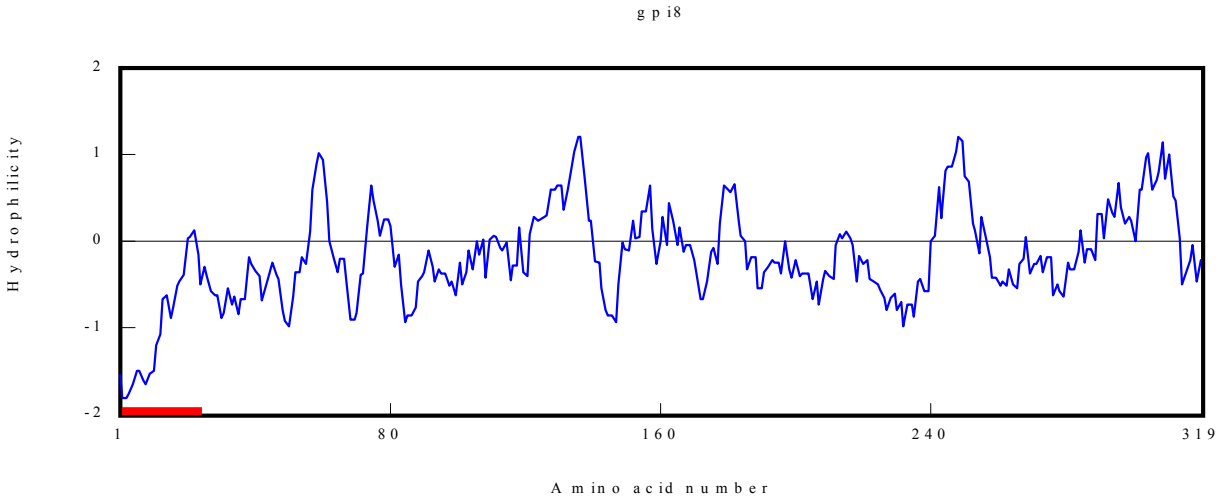

Fig. 3.16: Hydrophilicity Plot of the *Tb*Gpi8

The *Tb*Gpi8 has a putative hydrophobic N-terminal ER-directing signal sequence (underlined), but shows no hydrophobic membrane-spanning region at its C-terminus as compared with other Gpi8.

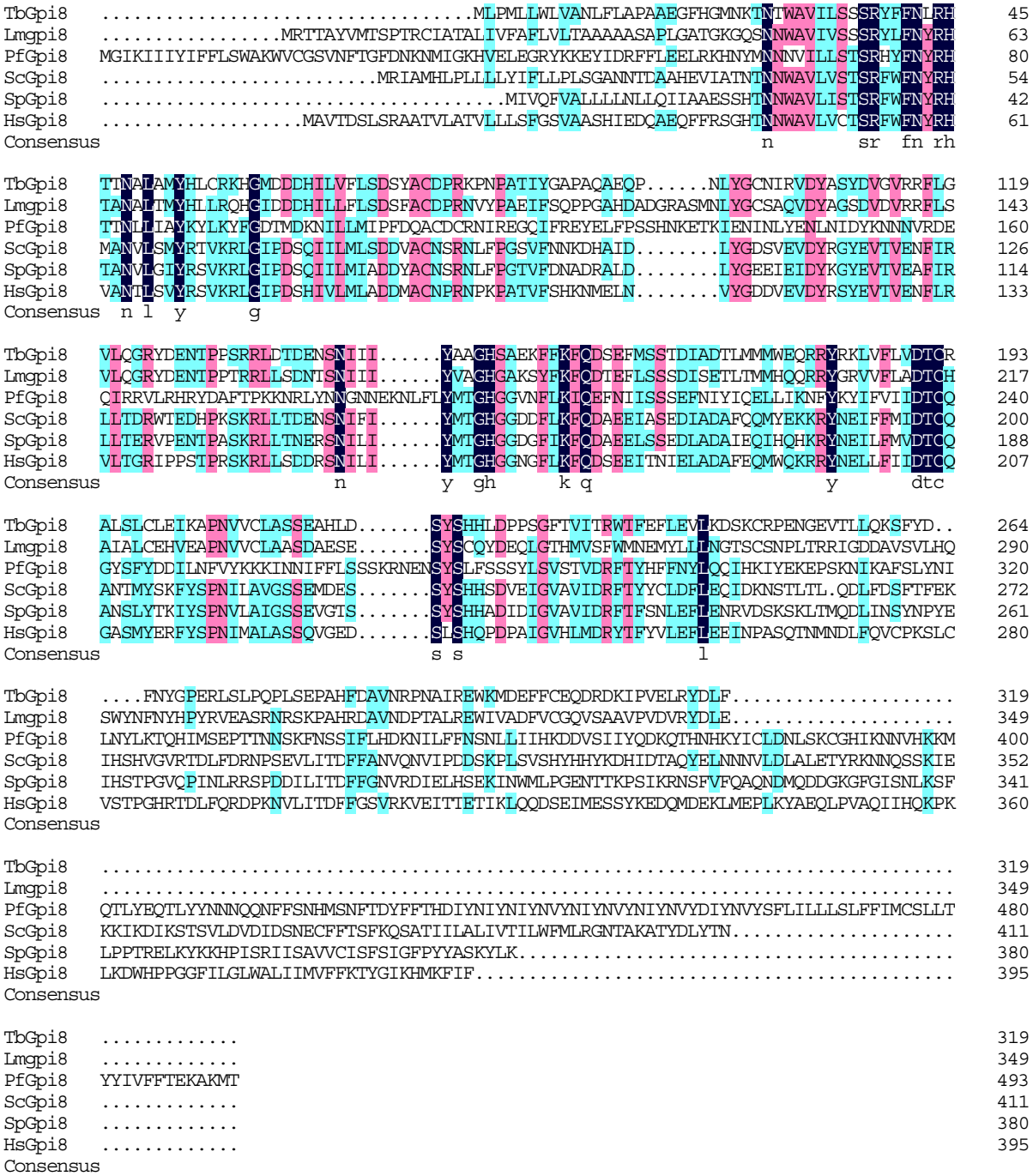

Fig. 3.17: Sequence Alignment of the *TbGPI8* with the Consensus Sequence of Other *GPI8*s

*Tb*Gpi8 protein sequence of *T. brucei* aligned with *L. mexicana*, *P. falciparum, S. cerevisiae, S. pombe* and *H. sapiens*. Amino acid identities and similarities are highlighted; conserved amino acid residues are labeled. One potential N-glycosylation site is at N^{25}(NKT), which is not conserved in other Gpi8.

As calculated from the amino acids sequence, *Tb*Gpi8 shows a pI of 5.51 and a molecular mass of 37 kDa or 35 kDa if the signal sequence is removed. Compared with other eukaryotic organisms, *Tb*Gpi8 shows a high degree of homology. According to the results of Meyer *et al*. (2000), *Sc*Gpi8 alleles mutated at Cys199 or His157 are non-functional, they are unable to suppress the lethality of Δgpi8 mutants. Alignment in Fig. 3.17 indicates that Cys193 and His142 of *Tb*Gpi8 are conserved and located at the consensus position of the Cys199 and His157 of *Sc*Gpi8. They should be the active site residues. The hydrophilicity plot of *Tb*Gpi8 (Fig. 3.16) shows one hydrophobic region at the N terminus, which may serve as a signal peptide for translocation into the ER. Unlike *Sc*Gpi8, *Tb*Gpi8 has no hydrophobic region at the C terminus.

3.4.2 Cloning of *TbGPI8* in Expression Plasmid

Two different expression systems were used to express *Tb*Gpi8. One was RGS6xHis tag system, which produced RGS6xHis-tagged *Tb*Gpi8, that, following expression, could be affinity purified on Ni-NTA matrices. The other was the MBP fusion protein system. The expressed fusion protein MBP-*Tb*Gpi8 could be affinity purified using amylose resin and further be digested with

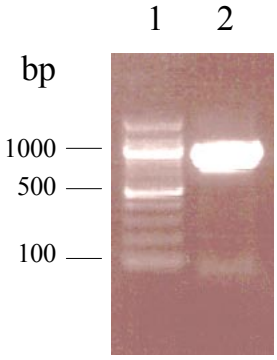

Fig. 3.18: Amplified *TbGPI8* in pBS KS$^+$
Lane 1: 1 kb marker
Lane 2: amplified *TbGPI8*

enterokinase to produce *Tb*Gpi8. To clone the *TbGPI8* into the expression vectors, the plasmid pBS KS$^+$ containing *TbGPI8* cDNA was used as a template. The plasmid pRSET B was used for the RGS6xHis tag system and plasmid pMAL-c2E was used for the MBP fusion system to heterologously express the *Tb*Gpi8. *TbGPI8* was amplified using primers GPI8-AS/GPI8-His-SE2 or GPSEMAL1/ GPASMAL2. By PCR, a DNA fragment of about 900 bp in size was produced (Fig. 3.18). This fragment did not contain the sequence for the N-terminal signal peptide.

For the 6xHis tag system the PCR product was digested using the restriction enzymes *Nhe*I/*Eco*RI and was ligated into the vector also digested with *Nhe*I/*Eco*RI. The RGS6xHis tag was located at the N-terminus of the same reading frame of *Tb*Gpi8.

For the MBP fusion system, the PCR product and the plasmid were both digested with *Kpn*I and ligated thereafter. The control PCR was performed using primer PMALSE1/GPASMAL2 to check for the correct orientation. *Tb*Gpi8 was placed at the C-terminus of MBP.

After transformation into *E. coli* 2566, all the plamids isolated from positive clones were restriction digested again to control correctness. In the RGS6xHis tag system, 6 positive clones were selected and digested with *Nhe*I/*Eco*RI. As shown in Fig. 3.19, 3 of them (clone 3, 4 and 8) produced the correct insert band while 3 others did not. Therefore, the 3 correct clones were used to express *Tb*Gpi8.

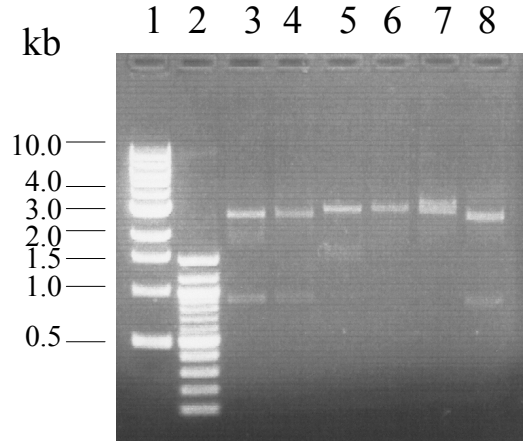

Fig. 3.19: *Nhe*I/*Eco*RI-Digestion of the Expression Vectors

Lane 1: 1kb marker
Lane 2: 100 bp marker
Lane 3-8: isolated plasmids from 6 different clones

3.4.3 Expression of *Tb*Gpi8 in *E.coli*

3.4.3.1 Induction and Purification of RGS6xHis-*Tb*Gpi8

For expression of *Tb*Gpi8, the *E. coli* strain containing plasmid pRSET B with *TbGPI8* was induced overnight with 0.3 mM IPTG at 15°C. Cells were lysed and centrifuged to separate supernatant and pellet. The supernatant was subjected to affinity purification using Ni-NTA matrices. The results of SDS/PAGE (Fig. 3.20) indicated that in the induced cells a protein with about

35 kDa was expressed at high level. This molecular mass corresponded to the molecular mass calculated from the *TbGPI8* sequence (lane 2), which was constructed in the expression plasmid pRSET B without the N-terminus. This protein could be affinity purified using a Ni-NTA matrix (lane 3), thus giving further evidence for the correct expression of *Tb*Gpi8. The RGS6xHis-*Tb*Gpi8s were expressed at high level, but most of them formed inclusion bodies. Although the RGS6xHis-*Tb*Gpi8s could be isolated from the supernatant in native form, the amount was very low as judged from SDS-PAGE. If the RGS6xHis-*Tb*Gpi8 was isolated in denaturing conditions, the protein seemed not bind to the Ni-NTA resin. Under these conditions, the 6xHis tag has probably degraded.

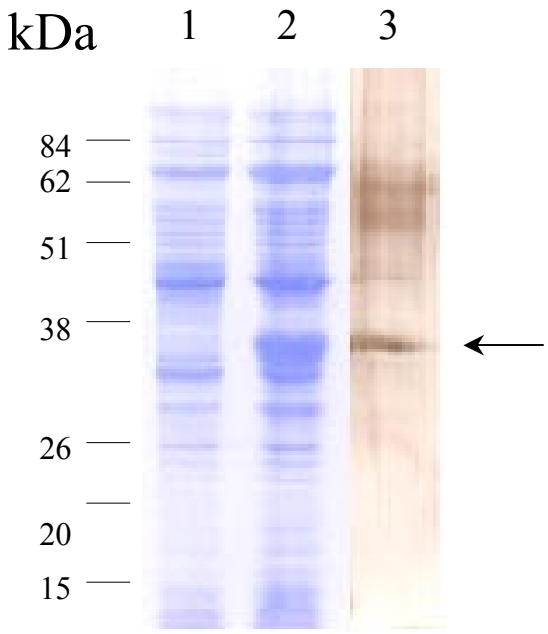

Fig. 3.20: Expression of RGS6xHis-*Tb*Gpi8 in *E. coli*

After induction of RGS6xHis-*Tb*Gpi8 in *E. coli* with 0.3 mM IPTG overnight at 15°C, the cell was sonicated in short pulses of 15 sec for 15 min each. The supernatant was applied to a Ni-NTA column. The RGS6xHis-*Tb*Gpi8 was eluted with elution buffer containing 250 mM imidazole. Lane 1: not induced cell; lane 2: with 0.3 mM IPTG induced cell; lane 3: using Ni-NTA matrices isolated *Tb*Gpi8

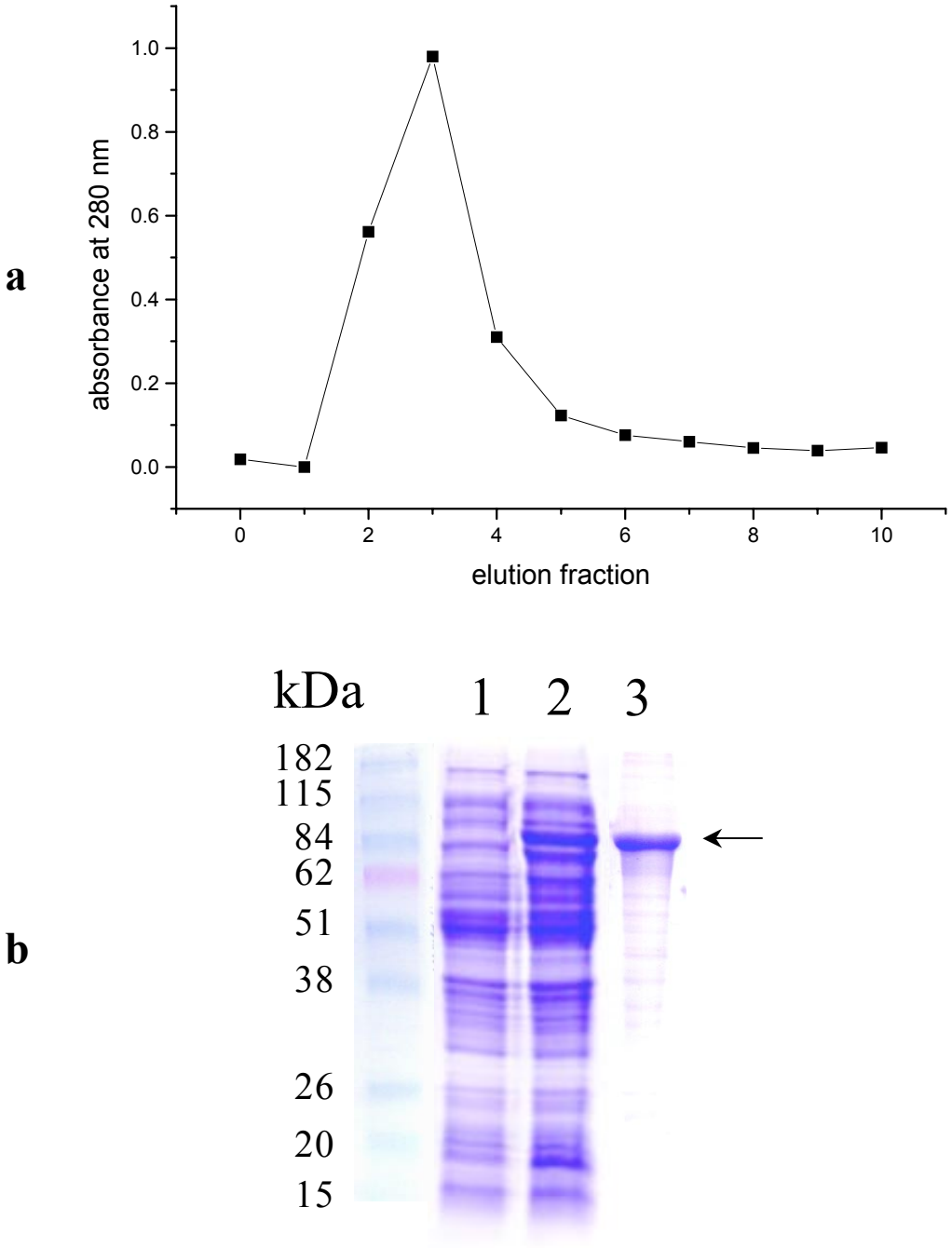

Fig. 3.21: Induction and Purification of MBP-*Tb*Gpi8

Cells were induced with 0.3 mM IPTG and sonicated in short pulses of 15 sec for 15 min. The supernatant was applied to an amylose column. The MBP-*Tb*Gpi8 was eluted with elution buffer containing 10 mM maltose. The protein concentration was measured at 280 nm. Panel a: Elution curve of MBP-*Tb*Gpi8; Panel b: SDS/PAGE analysis of MBP-*Tb*Gpi8. Lane 1: Lysate from not induced cells; lane 2: Cells induced with 0.3 mM IPTG; lane 3: Purified MBP-*Tb*Gpi8 using amylose resin.

3.4.3.2 Induction and Purification of MBP-*Tb*Gpi8

The *E. coli* strain containing *TbGPI8* in plasmid pMAL-c2E was induced overnight with 0.3 mM IPTG at 15°C. The cells were sonicated in short pulses of 15 sec each for about 15 min and centrifuged. The supernatant was supplied for affinity chromatography using amylose resin. As shown in Fig. 3.21, the MBP-*Tb*Gpi8 was induced by IPTG at high level. Following affinity chromatography, the MBP-*Tb*Gpi8 was purified as a single band on the SDS/PAGE showing a molecular mass of 80 kDa. From a 50 ml culture, about 2 mg of MBP-*Tb*Gpi8 was purified.

3.4.3.3 Cleavage of MBP-*Tb*Gpi8 Using Enterokinase

To obtain *Tb*Gpi8, enterokinase cleavage was carried out at a w/w ratio of 2.5%

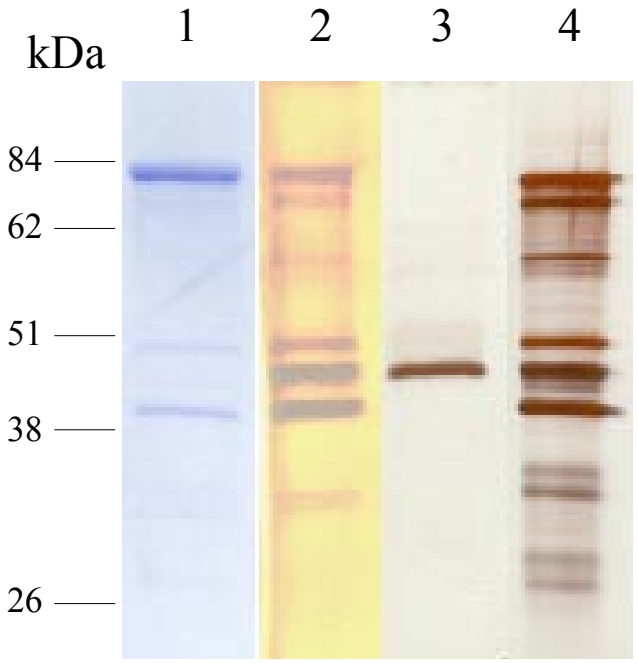

Fig. 3.22: Enterokinase Treated MBP-*Tb*Gpi8

Enterokinase cleavage was carried out at an enzyme/MBP-*Tb*Gpi8 (w/w) of 1/40 in Tris buffer, 50 mM, pH 8.0 at room temperature for 8 h. The cleavage mixture was subjected to an amylose column again to remove MBP from the *Tb*Gpi8. Samples were analysed by SDS/PAGE. Lane 1: Purified MBP-*Tb*Gpi8 before treatment with enterokinase; lane 2: MBP-*Tb*Gpi8 after treatment with enterokinase; Lane 3: *Tb*Gpi8 isolated from the cleavage mixture after a second run through the amylose column; lane 4: Elution buffer containing all retained protein bands from the amylose resin (cleaved MBP bound on amylose column).

~ 5% the amount of fusion protein. The reaction mixture was incubated overnight at room temperature. After cleavage with enterokinase, many different degraded products appeared on SDS/PAGE (Fig. 3.22, lane 2) and the MBP-*Tb*Gpi8 was not cleavage completely. This indicated that the fusion protein was not stable and that the enterokinase was not efficient to cleave the fusion protein. If the cleave mixture was applied to the amylose resin column again, the flow-though revealed only one protein band by silver staining (lane 3). This protein is supposed to be the pure *Tb*Gpi8, although its apparent molecular mass is a little higher than that calculated from the amino acid sequence. We assume that this protein consisted of the entire *Tb*Gpi8 containing some additional amino acids from the MBP, insufficient however to bind to the amylose resin. It is also possible that the standard MW marker was not very accurate under the employed electrophoresis conditions. The amylose resin column retained the maltose binding protein from the cleavage mixture successfully. As shown in Fig. 3.22, lane 4, almost all the degrading products were MBP linked, because they did not appear in the flow though of the amylose resin column, but were eluted using the corresponding elution buffer.

3.4.4 Fusion Protein GFP-C-TerminusVSG221

To measure the activity of the purified *Tb*Gpi8, a suitable substrate was needed. For this reason, a fusion protein GFP-C-terminusVSG221 was produced in *E. coli* using molecular cloning methodology. This protein consisted of the C-terminal sequence of MITat 1.2 linked to GFP. Since the C-terminal sequence contained the correct cleavage site, it was supposed to serve as a substrate for GPI anchor exchange. RT-PCR was performed on 221 bloodstream form cDNA with primers 221-PEPSE/221-PEPAS. The ~300 bp fragment was kinased and ligated to oligonucleotids 221-5ADA/221-3ADA to create the last codons of VSG 221 C-terminus and the sticky end compatible to *Sap*I cut pTYP1. The ligated product was treated with *Nde*I, purified by agarose gel electrophoresis and cloned into *Nde*I/*Sap*I treated pTYB1, rendering plasmid pTYB1::221PEP. EGFP gene was amplified with primers 3GFP-NDE/5GFP-NDE from plasmid pEGFP-N1. The product was cut using *Nde*I and cloned into *Nde*I treated and dephosphorylated pTYB1:: 221PEP. The primers 5GFP-NDE/221-PEPAS were used to check for the correct orientation of the fragment in the plasmid. After sequenced by GATC, the plasmids from correct clones were transformed into *E. coli* 2566 and induced overnight with IPTG at 15°C. Since the induced fusion

protein could not be visualized by Coomassie staining, Western blotting was performed using anti-C-terminusVSG221 antibodies to control the fusion protein. As indicated in Fig. 3.23 (lane 2), the induced fusion protein (about 95 kDa) was synthesized at a very low abundance (arrow a) but a protein of about 40 kDa was detected at a relative high level. These data indicate that the fusion protein intein-CBD-GFP-C-terminusVSG221 was degraded or cleaved *in vivo*. According to the molecular weight, this degraded protein of about 40 kDa might be the GFP-C-terminusVSG221. Further results (lane 3 and 4) suggest that most of GFP-C-terminusVSG221 stayed in the cell pellet but not in the cell supernatant. This was perhaps due to the highly hydrophobic C-terminus of VSG221.

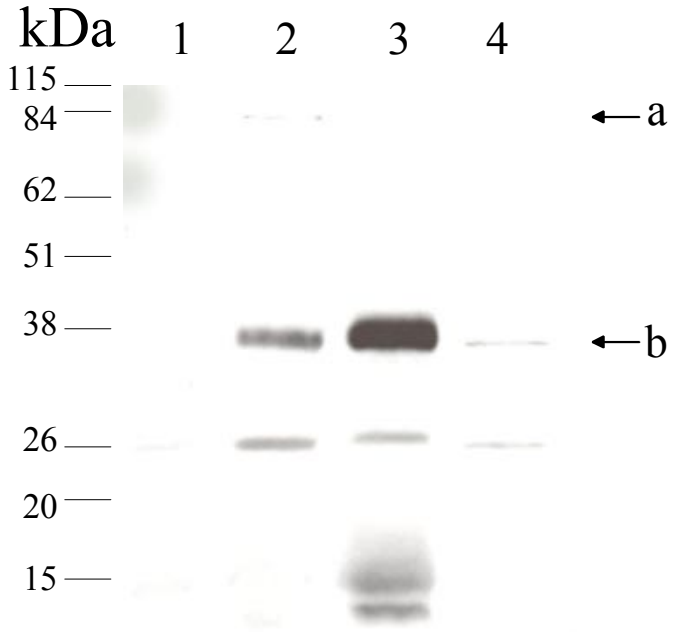

Fig. 3.23: Expression and Isolation of GFP-C-TerminusVSG221

Expression of the fusion protein was induced by 0.3 mM IPTG overnight at 15°C. Cell lysates were applied to SDS/PAGE and Western blotting analysis using anti-C-terminus VSG221 antibody. Lane 1: Lysate of not induced cells; lane 2: Lysate of induced cells; lane 3: Cell pellet; lane 4: Cell supernatant. Arrow a: Fusion protein intein-CBD-GFP-C-terminus VSG221; arrow b: Degraded GFP-C-terminus VSG221.

3.4.5 *In vitro* Activity Assay of the *Tb*Gpi8

3.4.5.1 *In vitro* Transamidase Assay Using GFP-C-TerminusVSG221 as Substrate

As shown in Fig. 3.23, most of the fusion protein intein-CBD-GFP-C-terminusVSG221 was already self cleaved *in vivo* into the target protein GFP-C-terminusVSG221, which was highly hydrophobic and remained in the pellet. If the supernatant contained some soluble intein-CBD-GFP-C-terminusVSG221 was applied to chitin column and conducted an on column cleavage, GFP-C-terminusVSG221 could not be eluted from column with moderate solution (data not show). Since it proofed difficult to isolate pure GFP-C-terminusVSG221, the corresponding *E. coli* lysate was directly be used as a substrate to test the activity of *Tb*Gpi8 *in vitro*. The anti-C-terminusVSG221 antibody was used for Western blotting to detect the C-terminus cleavage by *Tb*Gpi8. Trypanosomal lysate and enterokinase treated MBP-*Tb*Gpi8 as enzymes were incubated with *E. coli* lysate containing induced GFP-C-terminusVSG221 as substrate and hydrazine at 30°C for 1 h (Fig. 3.24).

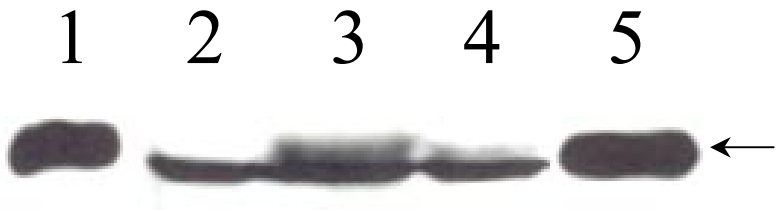

Fig. 3.24: *In vitro* Activity Assay of the *Tb*Gpi8

E. coli lysates containing the GFP-C-terminusVSG221 was incubated with trypanosomal lysate and enterokinase treated MBP-*Tb*Gpi8 at 30°C for 1 h. After SDS/PAGE, proteins were transferred onto a nitrocellulose membrane and detected with anti-C-terminusVSG221 antibodies. Lane 1: *E. coli* lysate containing induced GFP-C-terminusVSG221; lane 2: Trypanosomal lysate (This protein band showed cross reaction with anti-C-terminusVSG221 antibodies. It was not VSG); lane 3: *E. coli* lysate containing induced GFP-C-terminusVSG221 incubated with trypanosomal lysate; lane 4: *E. coli* lysate containing induced GFP-C-terminusVSG221 incubated with trypanosomal lysate and 10 mM hydrazine; lane 5: *E. coli* lysate incubated with enterokinase treated MBP-*Tb*Gpi8.

If the GFP-C-terminusVSG221 was incubated with trypanosomal lysate (lane 3), the C-terminus of the fusion protein was cleaved to some extent, because a relatively weak signal was obtained as comparing with the sample in the absence

of trypanosomal lysate (lane 1). The weakest signal was obtained by addition of 10 mM hydrazine (lane 4). Enterokinase treated MBP-*Tb*Gpi8 did not show any activity in this experiment (lane 5). These results suggest that the fusion protein GFP-C-terminusVSG221 is a suitable substrate for detection of the transamidase activity in lysates. In trypanosomal lysates, the transamidase complex could cleave the C-terminus from the fusion protein at high efficiency. However, cleavage of the C-terminus by heterogeneous expressed *Tb*Gpi8 could not be detected using this method even in the presence of hydrazine. Possibly, this method is not sensitive enough, because the minor decrease of substrate is normally difficult to be measured.

3.4.5.2 *In vitro* Transamidase Assay Using AMC Linked Peptide as Substrate

In this assay, Ac-S-V-L-N-AMC was used as substrate to check the transamidase activity of recombinant *Tb*Gpi8.

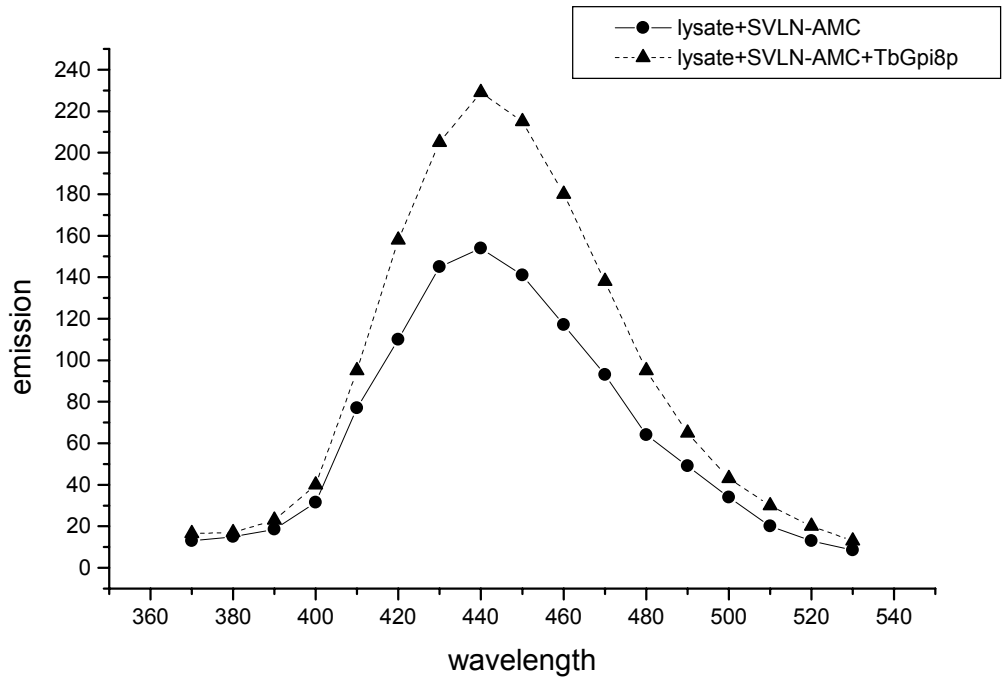

Fig. 3.25: Stimulation of the Transamidase Activity in Trypanosomal Lysates by *Tb*Gpi8

Trypanosomal lysate and substrate were incubated at 30°C overnight in the presence or absence of *Tb*Gpi8. The emission spectrum was measured in the range from 370 ~ 530 nm.

This method proofed to be much more sensitive than the method using GFP-C-terminusVSG221, because the amount of AMC produced by the transamidase activity was detected at nM level. In this assay, enterokinase treated MBP-*Tb*Gpi8 was first incubated with lysate and Ac-S-V-L-N-AMC overnight at 30°C in citrate buffer containing 10 mM hydrazine, pH 5.5. The transamidase activity was significantly stimulated by *Tb*Gpi8 (Fig. 3.25). The release amount of AMC was increased obviously. These results indicate that the recombinant *Tb*Gpi8 had some effect on the transamidase activity in trypanosomal lysates. According to the amino acid sequence of *Tb*Gpi8, it is expected to be a soluble protein. Therefore, addition of *Tb*Gpi8 to the cell lysate could increase the *Tb*Gpi8 concentration and then enhance the enzyme activity of the transamidase enzyme complex. GPI anchoring unit is a transamidase complex. Mutant GAA1 failed to express GPI-anchored proteins on the cell surface (Benghezal *et al*., 1996; Hamburger *et al*, 1995).

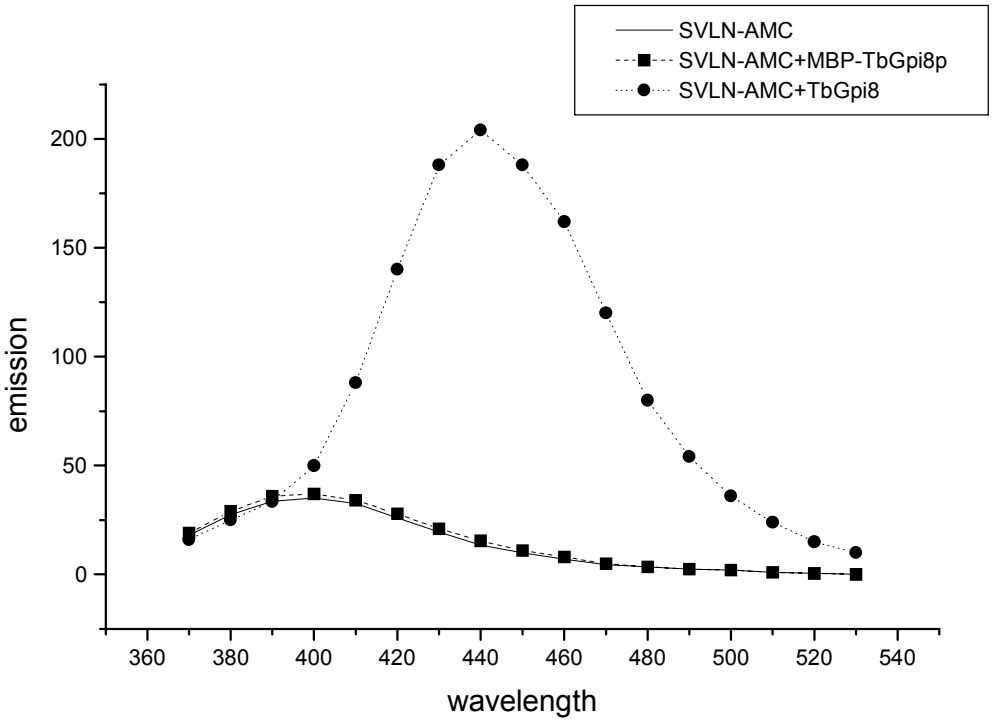

Fig. 3.26: Transamidase Activity of the Heterogeneous Expressed *Tb*Gpi8

*Tb*Gpi8 and MBP-*Tb*Gpi8 were incubated with substrate at 30°C overnight. The shift of emission maxima in samples was measured from 370 nm to 530 nm. Samples without *Tb*Gpi8 and MBP-*Tb*Gpi8 were also measured at the same conditions as control.

At least two gene products participated in the transamidation reaction. However, Gpi8 bears resemblance to several plant and invertebrate proteases. It could have some proteolytic activity itself. Based on this assumption, the transamidase activity of *Tb*Gpi8 was tested in the absence of trypanosomal lysate. As shown in Fig. 3.26, if the Ac-S-V-L-N-AMC was incubated with *Tb*Gpi8, the emission at 440 nm increased significantly as comparing with Ac-S-V-L-N-AMC incubated in buffer alone. The AMC was produced by enterokinase treated MBP-*Tb*Gpi8, whereas the AMC was not produced by intact MBP-*Tb*Gpi8. This result indicated that recombinant *Tb*Gpi8 has a peptidase activity itself and could cleave Ac-S-V-L-N-AMC at the P1 position, which corresponds to the ω position in VSG, leading to the GPI addition within the cell.

3.4.6 Anti-*Tb*Gpi8 Antibodies

3.4.6.1 Production and Determination of the Titer of Anti-*Tb*Gpi8 Antibodies

The MBP-*Tb*Gpi8 was heterologously expressed at high level but enterokinase cleavage was not very efficient and led to formation of many degraded proteins. *Tb*Gpi8 isolated from this mixture run at a relative high apparent molecular mass. Therefore, the fusion protein MBP-*Tb*Gpi8 was used as antigen to produce antibodies by immunisation of a hen. IgY was isolated from egg yolk by using PEG precipitation (See methods). The antibody titer was measured using ELISA with the respective antigen coated to plates. A titer of 1:100,000 each 10 ml preparation from 3 yolks was achieved.

3.4.6.2 Purification of the Specific Anti-*Tb*Gpi8 IgY

The fusion protein MBP-*Tb*Gpi8 was used to produce anti-*Tb*Gpi8 antibodies. By using PEG precipitation a mixture of IgYs against different antigens was isolated. (Fig. 3.27, lane 1). In order to obtain pure anti-*Tb*Gpi8 IgY, anti-MBP IgY was removed from antibody mixture by using MBP coupled to an amylose column. Anti-*Tb*Gpi8 IgY was then further purified using an MBP-*Tb*Gpi8-amylose column from the flow through (Fig. 3.27, lane 2). In order to check the specificity of the anti-*Tb*Gpi8 IgY, *E. coli* strain with pTYB1::MBP was used in Western blot analysis. The synthesis of MBP was induced by 0.3 mM IPTG. If the IgY mixture isolated from egg yolk was not subjected to further purification

by using affinity chromatography, it gave a very strong signal on the Western blot by the induced MBP (Fig. 3.28, panel a). After purification, induced MBP

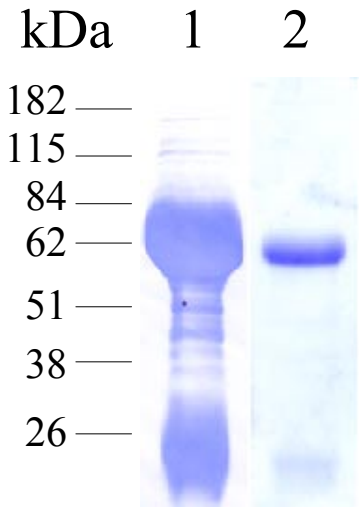

Fig. 3.27: IgY Isolated by PEG Precipitation and Affinity Chromatography

IgY was precipitated from egg-yolk by using 12% PEG6000 (lane 1) and subjected to affinity chromatography using two columns with MBP and MBP-*Tb*Gpi8 (lane 2). After SDS/PAGE, proteins were visualized with Coomassie staining.

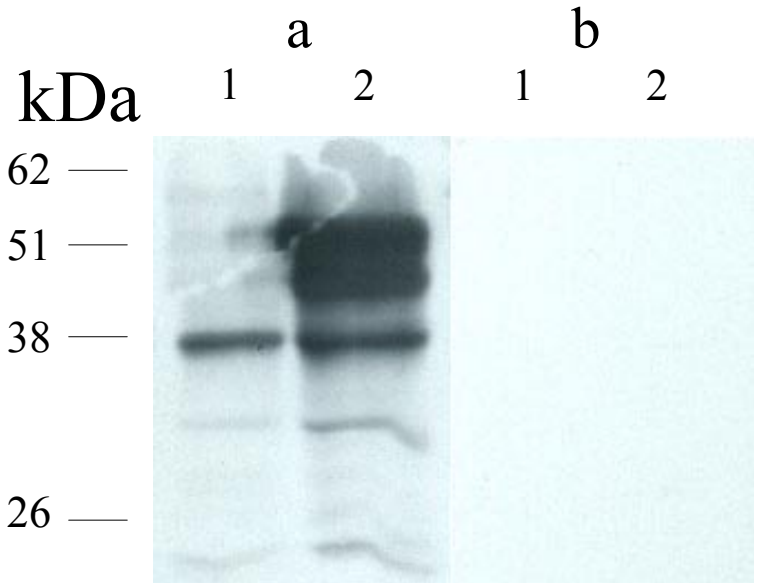

Fig. 3.28: The Specificity of the Purified Anti-*Tb*Gpi8 IgY

E. Coli cells containing MBP gene were incubated in the presence (lane 2) and absence of 0.3 mM IPTG (lane 1). After SDS/PAGE, proteins in induced cells and not induced cells was transferred on to nitrocellulose membrane and detected with PEG 6000 precipitated IgY (panel a) and affinity purified IgY specific against TbGpi8 respectively (panel b).

could not be detected on the plot (Fig. 3.28, panel b). This result indicated that the anti-MBP IgY was removed from the IgY mixture efficiently by the affinity chromatography. Although the anti-*Tb*Gpi8 IgY was affinity purified, it had still little cross-reaction with an unknown protein in *E. coli.*

3.4.7 Localization of *Tb*Gpi8 Using Isolated Anti-*Tb*Gpi8 IgY

The localization of *Tb*Gpi8 was performed using anti-*Tb*Gpi8 IgY. Cell nucleus and kinetoplast were labelled with bisbenzimid. The results are shown in Fig. 3.29. In the procyclic cell (panel a), clearly stained vesicles are shown to lie between nucleus and kinetoplast. This position is known to represent the Golgi apparatus. In bloodstream form cells, similar vesicles were shown to lie not only between nucleus and kinetoplast but also closely behind the nucleus.

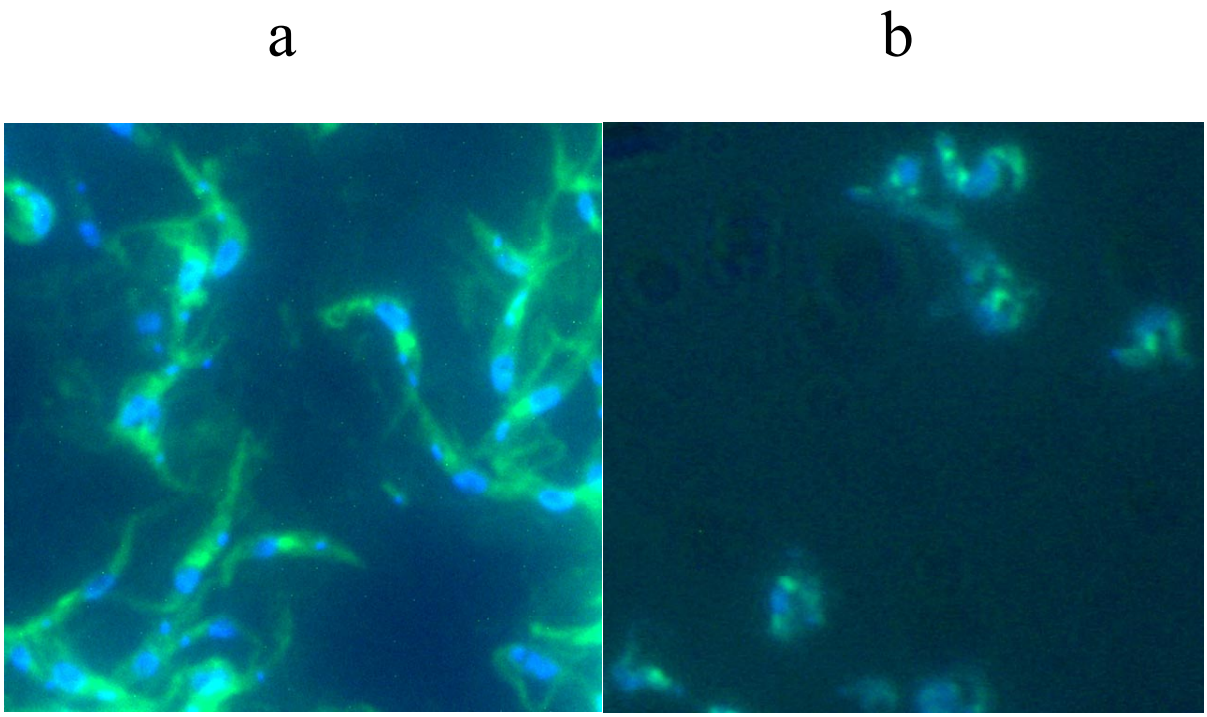

Fig. 3.29: Localization of the *Tb*Gpi8

Cells in procyclic form (panel a) and in bloodstream form (panel b) were fixed with 1% formaldehyde in HBS buffer, and then the localization of the *Tb*Gpi8 was visualized by specific anti-*Tb*Gpi8 antibody developed from chicken. The second antibody was FITC labeled anti-chicken IgG.

3.4.8 Localization of the Pro-VSG

It was presumed that replacement of the C-terminal signal sequence with GPI moiety is catalysed by an ER-localized transamidase rapidly after translation (Mayor & Maxfield, 1995; Maxwell *et al*., 1995). According to the results presented in 3.1.4, in the trypanosomal lysate exists some pro-VSG, which could be detected by anti-C-terminus antibody. The results of 3.4.7 indicate the main component of transamidase was located in Golgi but not in ER. It is possible that the in ER newly formed pro-VSG must transported to the Golgi to finish anchor exchange, which caused some pro-VSG accumulated in ER. In order to check this assumption, the localization of pro-VSG was performed by immunofluoresence using anti-C-terminus antibodies. As shown in Fig. 3.30, the most of the pro-VSG was localized in the ER (panel b), which emerge from the nuclear envelope and ramify through the cytoplasm. These results confirmed the Golgi localization of *Tb*Gpi8 and allowed to explain the existence of a steady state pool of pro-VSG.

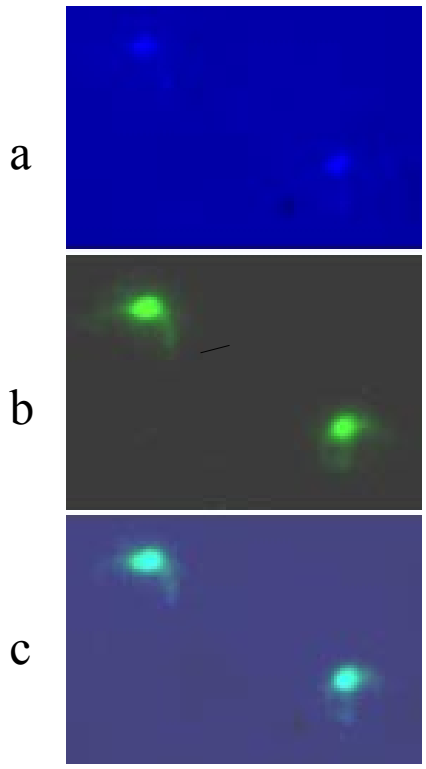

Fig. 3.30: Localization of the Pro-VSG

Trypanosomal cells in bloodstream form were fixed with 1% formaldehyde in HBS buffer. Anti-C-terminusVSG221 antibody was used to localize pro-VSG within the cell. The second antibody was FITC labeled anti-rabbit IgG. Panel a: Nucleus and kinetoplast labelled with bisbenzimid; panel b: Cells labelled with anti-C-terminus antibody; panel c: Overlay of panel a and panel b.

3.4.9 Analysis of Trypanosomal Lysate Using Isolated Anti-*Tb*Gpi8 IgY

The sequence of *Tb*Gpi8 predicted a protein containing 302 amino acids and a molecular mass of 37 kDa or 35 kDa if the signal sequence is removed. The hydrophilicity plot of the *Tb*Gpi8 (Fig. 3.31) reveals one hydrophobic region at the N terminus, which qualifies as a signal peptide for translocation into the ER (von Heijne, 1986). The cleavage site for the signal peptidase is predicted between the residues Ala 17 and Ala 18. *Tb*Gpi8 in trypanosomal lysate was detected by SDS/PAGE and Western blotting using affinity purified anti-*Tb*Gpi8 IgY. As shown in Fig. 3.31, this antibody specifically detected 3 bands of ~37 kDa, ~35 kDa and ~33 kDa in trypanosomal lysates. According to the molecular weight and amino acids sequence, *Tb*Gpi8 contains a N-terminal signal sequence and a N-glycosylation site. The N-terminal signal sequence should not be detected because it is cleavaged during the translation. The size difference between the 35 and 37 kDa form of *Tb*Gpi8 may be due to glycosylation. The state of glycosylation of *Tb*Gpi8 has not been investigated yet. This ~33 kDa band may represent a degradation product or be labelled non-specifically by our antibodies.

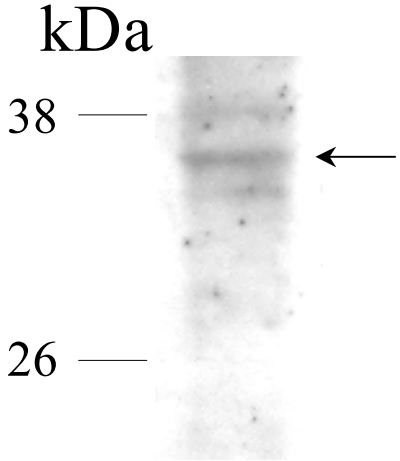

Fig. 3.31: *Tb*Gpi8 in Trypanosomal Lysate

Trypanosomal lysate was separated by SDS/PAGE and analysed with affinity purified chicken anti-*Tb*Gpi8 IgY by Western blotting.

4 Discussion

4.1 The *in vitro* Translation System

By using limited lysis under hypotonic condition, which just led to cell lysis but not lysis of organelles, a potent small molecular mass translation inhibitor was prevented from being released into the cell lysate and a high translation activity was obtained. However, these translationally active lysates of trypanosomes had no reinitiation activity. The main problem seems to be phosphorylation of crucial initiation factors like eIF-2 or eIF-4. Regulation of translation initiation is an important component of the cellular stress response. By preparation of cell lysates, several factors (temperature, pressure, pH changes, etc.) appeared to invoke the stress-mediated regulation of eIFs to alter the translational capacity by phosphorylation. This mediates the binding of the initiator Met-tRNA to the ribosome or recruits mRNAs to the ribosome by recognition of the 5'-m7G cap and serves as a central adapter by binding to various translation factors and regulators (Sheikh *et al*., 1999). Nevertheless, this cell free system is very useful as it allows the study of post-translational modifications *in vitro.*

VSG of the *T. brucei* bloodstream form is known to be glycosylated leading to a range of structures including high mannose and complex types. This work provided evidence for *in vitro* N-glycosylation. N-glycosidase F treatment and concanavalin A blotting clearly revealed a coupling of *in vitro* translation and *in vitro* modifications. The cotranslational translocation into the ER-lumen is an essential prerequisite for N-glycosylation. Therefore, demonstration of N-glycans is a good indicator for translocation of newly formed proteins into the ER-lumen *in vitro*. Upon mixing trypanosomal lysate with reticulocyte lysate and reinitiation of a translation, the newly formed proteins did not undergo any modification, i.e. reinitiation and translation happened in reticulocyte lysate but a coupling with trypanosomal ER membranes and translocation into trypanosomal ER was not achieved.

Within trypanosomal lysates pro-VSG containing the uncleaved C-terminus could be regularly detected no matter whether *in vitro* translation was conducted or not. Among the characterized GPI proteins, the number of residues within the COOH-terminal signal peptide varied from about 10 to 30 mainly hydrophobic

amino acids. In trypanosomes it would be exchanged by GPI anchor within 1 min of completion of the polypeptide (Ferguson *et al*., 1986). Our data suggest that the processing was delayed or incomplete and pro-VSG accumulated. According to Kodukula (1991), processing of pro form miniPLAP to the mature form was also delayed considerably. After 90 min, only approximately 40% of the total translated material appeared as mature miniPLAP. A possible reason for this lag is the amount of COOH-terminal transamidase activity within the ER membrane. In our experiments, the appearance of pro-VSG is perhaps due to a relative low amount of COOH-terminal transamidase in ER as compared with the Golgi apparatus but this will be considered later.

Our main interest to develop a cell free system was to study the addition of GPI anchor, which is thought to be a single-step reaction performed by a transamidase (Udenfriend *et al*., 1995). As VSG is a dominant protein in trypanosomes, accounting for about 10% of the total amount of proteins and is constitutively synthesized at a high rate (about 8% of total protein biosynthesis), this parasite seems to be perfectly suited for such studies. There are several methods to determine the GPI anchor. Due to myristate exchange (reactions occurring on mature VSG), GPI labeling with tritiated myristic acid could not be used to investigate GPI anchor exchange. Radiolabeling with either GDP-[^{14}C]mannose or [^{14}C]ethanolamine could be detected but was rather poor probably due to a substantial amount of preformed GPI precursor molecules within the microsomal membranes. Due to the high activity of endogenous GPI-PLC, phase separation with Triton X-114 is not suitable for detection of the GPI anchor, because newly formed mfVSG was transformed to sVSG very quickly. $ZnCl_2$, an inhibitor of GPI-PLC, could inhibit the enzyme only partially. Some other inhibitors, however, had a notable impact on translation. For these reasons, two important substances were used for the measurement of the anchor addition *in vitro*. One is biotin-lys-tRNA^{lys}, which allows to purify newly formed VSG from the translation mixture and to eliminate the interference of ‘old’ VSG. In VSG there are more lysine residues than methionine. By using an ECL fluorescent detection kit the newly translated proteins were easily detected. This proofed to be a very sensitive and timesaving method for *in vitro* translation. The other was the use of anti-CRD antibodies. These antibodies have been originally prepared from *Leishmania* cells and show no cross reactivity with the respective VSG variant as confirmed by using isolated mfVSG. The purified newly formed VSG could be detected with anti-CRD antibodies, clearly showing that the GPI is added to the premature VSG molecule *in vitro*. In

addition, some newly formed VSGs were kept in membrane form in the presence of 0.2 mM $ZnCl_2$, and the VSG of MITat 1.4 could be labelled by using UDP-[^{14}C]galactose, which also provided direct or indirect evidence for the GPI anchor exchange capability *in vitro*.

4.2 *In vivo* Research of GPI Addition to VSG

The mature GPI anchor precursor is added to the carboxyl-terminus of protein *via* an amide bond formed between the carboxyl-terminus and ethanolamine. A carboxyl-terminal sequence signals its own cleavage and replacement with the GPI anchor. This replacement reaction is though to be a transamidation (Udenfriend *et al*., 1995). In a cell-free system, addition of hydrazine resulted in the linkage of hydrazine in place of the GPI-anchor forming a hydrazide of miniPLAP. In the work presented here, trypanosomes MITat 1.2 were cultured in the presence of 10 ng/ml tunicamycin to produce nonglycosylated VSG. This VSG runs relatively faster in SDS/PAGE than glycosylated VSGs and allows to be analysed further without interference of glycosylated VSG. VSG MITat 1.2 has two N-glycosylation sites. Appearance of additional 2 VSG bands with lower molecular weight may be due to different N-glycosylation, the VSG with just one glycosylated site and the nonglycosylated VSG. After Western blotting analysis, just the lowest band could be detected by anti-CRD antibodies whereas the other could not. After translation and processing, many C-terminal forms may be formed and could not be characterized by anti-CRD antibodies, such as mfVSG, free VSG (without anchor), pro-VSG (with C-terminal signal peptide). This needs to be further characterized. Nevertheless, my experiments have successfully given evidence that small nucleophiles are capable of substituting for GPI in the anchoring reaction, because in the presence of hydrazine the produced VSG could not be detected by anti-CRD antibody and in the presence of biotin-hydrazine the biotin tag on VSG could be detected by streptavidin.

The GPI anchor appears to act as a signal to target proteins to the apical portion of the plasma membrane (Lisanti *et al*., 1989; 1990) and may also function in the export of proteins from the ER. Disruption of GPI attachment, either in cell lines that are deficient in GPI precursor or by mutation of GPI-peptides, can result in the retention of mammalian GPI-anchored proteins in the ER (Moran *et al*., 1992; Field *et al*., 1994). McDowell *et al*. (1998) have investigated the transport of VSGΔgpi. Their studies also indicated that the GPI-minus VSGs

accumulate in the endoplasmic reticulum (ER). The delayed forward transport cannot be accounted for by failure to fold/assemble in the absence of a GPI anchor. The GPI structure acts in a positive manner to mediate efficient forward transport of some, and perhaps all, GPI-anchored proteins in the early secretory pathway of trypanosomes. Therefore, in my experiment, if the GPI anchor was replaced by a small nucleophile, similar results were expected. Immunolocalization of VSG indicated that VSG-hydrazide accumulated in several extensively stained compartments between nucleus and kinetoplast. Obviously they do not accumulate in the tubular structures of ER. Normally, VSG is transported along the classical intracellular route for glycoproteins and is delivered to the flagella pocket, where it is integrated into the surface coat (Duszenko *et al*., 1988). Accumulation of a protein within ER or Golgi can occur for different reasons, including improper folding, absence of exit signals, or recycling. A possible explanation for my results is that GPI-minus VSG, i.e. VSG-hydrazide, was arrested within the Golgi and trans-Golgi region and the incorporation of newly synthesized VSG into the surface coat was highly retarded. As compared with results obtained by McDowell et al. (1998) using VSGΔgpi, we found a different localization for the accumulated VSG-hydrazide. The VSGΔgpi was synthesized as a C-terminus-minus VSG within the ER and thus the retention begins already in the ER. In my experiments, VSG was synthesized as a pro-VSG in the ER as usual, which has a C-terminal peptide as membrane anchor. This peptide could mediate efficient transport from ER to Golgi apparatus where the pro-VSG was transformed to VSG hydrazide in the presence of hydrazine and the transamidase complex. From now on, VSG existed as a GPI-minus VSG and could not be exported to the cell-surface efficiently. The localization of the putative transamidase will be discussed later.

4.3 The Transamidase Activity in Trypanosomal Lysates and the Synthetic Ac-S-V-L-N-AMC Substrate

The substrate Ac-S-V-L-N-AMC was quite efficiently cleaved at the P1 position in trypanosomal lysates and the fluorogenic AMC was released. This reaction could be activated by 10 mM hydrazine. Hydrazine is a well-known nucleophilic acceptor in transamidase and transpeptidase catalysed reactions (Tate *et al*., 1974). Therefore, we may suppose this reaction is performed by a transamidase in trypanosomal lysates. Additionally, enzyme activity was inhibited by 1 mM

pCMPSA, a sulfhydryl alkylating reagent, completely. This result is consistent with the proposal that the trypanosome GPI transamidase contains a catalytically important sulfhydryl residue. About 30 families of peptidases are dependent on a cysteine residue at the active centre (Rawlings and Barrett, 1994). Most protozoa produce cysteine endopeptidases during at least one stage of their life cycle. Most of them are members of the papain super family. These are predominantly lysosomal enzymes, which do not show substrate specificity for asparaginyl residues. They have a preference for bulky hydrophobic residues at the P2 position such as Val and Phe (Harris *et al*., 2000). Therefore, the proteolytic activity detected using Ac-S-V-L-N-AMC as a substrate can be due to the transamidase activity of *Tb*Gpi8, which has a high homology to other known Gpi8 and C13 cysteine peptidases. Legumain is a C13 cysteine endopeptidase that shows a significant homology with Gpi8. Its maximal activity is seen at pH 5.8 under assay condition (Chen *et al*., 1997). The optimum pH of the transamidase in trypanosomal lysates in my experiments was at pH 5.5. These data suggest that the transamidase activity detected in trypanosomal lysates is due to *Tb*Gpi8, which may function better in acidic condition than neutral condition. However, at present there is not enough evidence to confirm that these assumptions are valid *in vivo*.

4.4 Cloning, Expression and Characterization of *Tb*Gpi8

Transamidase-deficient cells are expected to accumulate complete GPIs as well as the GPI precursor proteins. This phenotype is exhibited by two yeast mutants, gaa1 and gpi8 and a mammalian mutant cell line (class K) (Mohney *et al*., 1994; Benghezal *et al*., 1995; Hamburger *et al*., 1995). Benghezal *et al*. (1996) have cloned the GPI8, which encodes a transmembrane ER protein with a large luminal domain. Yeast Gpi8 has 27.5% identity to a jack bean asparaginyl endopeptidase, which shows transpeptidase activity *in vitro* (Abe *et al*., 1993). They belong to a novel cysteine proteinase family. The homology with proteases does not prove, but at least suggests that Gpi8 is directly involved in the proteolytic removal of the GPI anchoring signal.

To gain a better understanding of the properties of *Tb*Gpi8, *TbGPI8* cDNA was cloned and *Tb*Gpi8 was expressed and characterized. It showed significant homology with other known *GPI8* genes. The size of the cDNA is very similar to the *LmGPI8*. A hydropathy analysis showed that *Tb*Gpi8 has a N-terminal

hydrophobic domain that is likely to serve as a signal for ER localization. It can be assumed that *Tb*Gpi8 is glycosylated *in vivo* as it contains the signal peptide. According to the amino acids sequence, *Tb*Gpi8 contains one N-glycosylation site, being on Asn 25, which is close to the most probable cleavage site for the signal peptidase (von Heijne, 1986). Western blotting analysis of trypanosomal lysates with specific anti-*Tb*Gpi8 antibodies also suggests this assumption of N-glycocylation.

Like *Lm*Gpi8, *Tb*Gpi8 lacks a C-terminal hydrophobic domain found in yeast and human GPI8s that is predicted to be a transmembrane helix (Benghezal *et al*., 1996). Human Gpi8 mutant lacking the transmembrane domain retained its activity to complement class K mutant cells, indicating that the transmembrane domain is not necessary (Ohishi *et al*., 2000). For the transamidase activity, however, the GPI8 protein should be anchored to the luminal leaflet in order to interact with the preformed GPI anchor. Hilley *et al*. (2000) suggested that attachment of *L. mexicana* GPI8 to the ER membrane may thus require one or more other integral membrane proteins. A possible candidate is Gaa1p. The exact role of Gaa1p was not elucidated yet, but it had been demonstrated that Gaa1p and Gpi8 form a protein complex (Ohishi *et al*., 2000). In human and yeast cells, Gaa1p is a luminally oriented ER glycoprotein containing several transmembrane domains (Hamburger *et al*., 1995). It is possible that Gaa1p is required for a precursor protein to form a carbonyl intermediate with the GPI transamidase. Ohishi *et al*., (2000) showed GAA1 knockout cells were defective in the formation of carbonyl intermediates between precursor proteins and the transamidase. We have also found a *TbGAA1* gene by using the BLAST program and TIGR-Database, which has a high homology with hGAA1 and yGAA1 and showed several transmembrane domains. It needs to be further characterized.

Cellular localization using specific antibodies shows that *Tb*Gpi8 is mostly localized in the Golgi apparatus. Some other results, such as pro-VSG, which is mostly found within the ER, and VSG-hydrazid, which is transported from ER to the Golgi apparatus but not further to the plasma membrane, also provided some evidence for the localization of transamidase within the Golgi apparatus. Resident Golgi proteins must have localization signals to ensure that they are targeted to the correct Golgi compartment and not swept further along the secretory pathway. It may also be supposed that within the trypanosomal Golgi apparatus, the *Tb*Gaa1p form an enzyme complex with *Tb*Gpi8. Gpi8 contains

no KKXX and KDEL retrieval motifs, which are necessary and sufficient for the recycling of proteins from the Golgi complex to the ER. Lotti *et al*. (1999) indicated that proteins tagged with the KKXX motif are located along the entire early segment of the secretory pathway, *i.e.* ER, IC, and cis-Golgi complex, but are not confined within the ER. Some of them have been found located in the Golgi complex at steady state. The localization of *Tb*Gpi8 within the Golgi complex may also be mediated by such a protein with KKXX motif. Recent studies indicate that there are a number of different Golgi localization signals and mechanisms for retaining proteins within the Golgi apparatus such as oligomerisation, lipid-mediated sorting and intra-Golgi retrograde transport (Gleeson, 1998). More information is required to determine the Golgi localization of *Tb*Gpi8.

It is believed that Gpi8 is the catalytic subunit (Yu *et al*., 1997). This protein shares significant homology with a family of previously characterized cysteine proteinases, the asparaginyl endopeptidases known as legumains (Benghezal *et al*., 1996), which were originally identified in the seeds of leguminous plants (Ishii, 1994). These proteins have been categorized as family C13 of cysteine peptidases (Riezman and Conzelmann, 1998). The GPI:protein transamidases belong to family C13 of the cysteine peptidases (Riezman and Conzelmann, 1998) and they are likely to possess the classical catalytic dyad residues, cysteine and histidine, that mediate their activity. It has been shown that GPI:protein transamidase activity is susceptible to sulfydryl alkylating agents, implying that the protein has an essential cysteine (Sharma *et al*., 1999). This study of *Tb*Gpi8 in trypanosomal lysates showed also the efficient inhibition of the transamidase activity by pCMPSA. Two cysteine (Cys76 and Cys192, *Tb*Gpi8 numbering) and two histidine (His45 and His150) residues are conserved among trypanosomal, leishmania, yeast, and human GPI8 (Fig. 3.16). Both histidine residues are also conserved in legumain, whereas only one of the cysteine residues is conserved across all C13 family members (Cys192, *Tb*Gpi8 numbering). Thus, this residue is the prime candidate for the active site cysteine. Meyer *et al.* (2000) proved that Cys199 and His157 in *Sc*Gpi8 are the active site residues. In *Tb*Gpi8, it is most likely that the two corresponding residues, Cys192 and His150, are the active site.

The detected enzyme activity of the heterologously expressed *Tb*Gpi8 suggests that the enzyme is in its mature form, despite of its different apparent molecular mass. This difference might be due to an incorrect cleavage of MBP-*Tb*Gpi8 by

the used enterokinase or to a slightly different folding. Use of the recombinant *Tb*Gpi8 and the developed specific antibodies have provided important tools for the investigation of the transamidase mechanism of GPI anchoring, and the organization and enzymology of the transamidase complex.

Summary

The parasitic protozoan *Trypanosoma brucei* expresses a variant surface glycoprotein (VSG), which protects it from lysis by host serum components. All mature VSGs are N-glycosylated and glycosylphosphatidylinositol (GPI) anchored.

To study trypanosome-specific post-translational modifications of VSG, a cell-free system was produced by 'limited lysis' of bloodstream forms of *Trypanosoma brucei*. Newly formed proteins were labelled using [^{35}S]methionine or biotinylated lysine. After *in vitro* translation and N-glycosidase F treatment, a VSG with a slightly smaller apparent molecular mass appeared, indicating that newly synthesized VSG underwent translocation and glycosylation in this cell-free system. Biotinylated proteins were isolated by affinity chromatography from *in vitro* translation samples by using immobilized avidin. Specific anti-CRD antibodies were used successfully to demonstrate the existence of the GPI anchor on newly formed VSG. Appearance of mfVSG by inhibition of endogenous GPI-PLC with $ZnCl_2$ and labeling of VSG with UDP-[^{14}C]galactose provided conclusive evidences for the linkage between *in vitro* translation and post-translational modification in our cell free system.

It was supposed that GPI anchoring was processed by a transamidation reaction (Udenfriend *et al*., 1995). The transamidase mechanism of GPI anchoring was studied by culture of bloodstream form *Trypanosoma brucei* in media containing hydrazine or biotinylated hydrazine. Tunicamycin was also used in the media to form deglycosylated VSG, which allows analysis without interference of old VSG. In the presence of hydrazine, newly formed VSG was not detected with anti-CRD antibodies and in the presence of biotinylated hydrazine, newly formed VSG was detected by streptavidin, indicating that hydrazine or biotinylated hydrazine functioned as substitutes of GPI and thus covalently linked to the C-terminus of VSG. Intracellular localization of VSG in the cells cultured in the presence of hydrazine demonstrated that VSG was not transferred to the cell surface efficiently, because there are a few vesicles between cell nucleus and kinetoplast, which could be visualized by anti-VSG antibody very intensely. These results suggest the incorporation of nucleophilic agents during the transamidation reaction. Hence, the GPI anchor addition of VSG is catalysed

by a transamidase. Study of the transamidase activity in cell lysates using the synthetic substrate Ac-S-V-L-N-AMC showed a pH optimum of 5.5 and the activity could be stimulated by hydrazine and inhibited by pCMPSA, suggesting not only a transamidation reaction mechanism of the GPI anchoring but also a functionally sulfhydryl residue in the active centre of the transamidase.

*Tb*Gpi8 was cloned and expressed in *E. coli. TbGPI8* encodes for a 37 kDa or 35 kDa protein if the signal sequence is removed. It has one potential N-glycosylation site near the N-terminus. *Tb*Gpi8 shows a significant homology to other Gpi8ps, which belong to a novel cysteine protease family. Like *Lm*Gpi8, *Tb*Gpi8 has no C-terminal hydrophobic region. Homology between this family and other Cys proteinases, such as caspases, pointed to Cys192 and His150 as potential active site residues. Enzyme activity assays using heterologously expressed *Tb*Gpi8 and Ac-S-V-L-N-AMC showed cleavage of the substrate, indicating that Gpi8p is indeed directly involved in the proteolytic removal of the GPI anchoring signal. Intracellular localization of the *Tb*Gpi8 within the Golgi apparatus was observed by using specific anti-*Tb*Gpi8 antibodies, which were developed from chicken. This result allows to explaining the existence of pro-VSG within the ER and a transport block of VSG hydrazid from Golgi to cell surface. Western blotting analysis using anti-*Tb*Gpi8 antibodies demonstrated 3 protein bands of 37, 35 and 33 kDa. The 37 kDa band should be N-glycosylated *Tb*Gpi8.

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