GPI anchor transamidase of *Trypanosoma brucei*: in vitro assay of the recombinant protein and VSG anchor exchange

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Summary

***GPI8* from *Trypanosoma brucei* was cloned and expressed in *Escherichia coli*. *TbGPI8* encodes a 37 kDa protein (35 kDa after removal of the putative signal sequence) with a pI of 5.5. It contains one potential N-glycosylation site near the N-terminus but no C-terminal hydrophobic region. Enzyme activity assays using trypanosomal lysates or recombinant *Tb*Gpi8 exhibited cleavage of the synthetic peptide acetyl-S-V-L-N-aminomethyl-coumarine, indicating that *Tb*Gpi8 is indeed directly involved in the proteolytic processing of the GPI anchoring signal. Intracellular localization of *Tb*Gpi8 within tubular structures, such as the endoplasmic reticulum, was observed by using specific anti-*Tb*Gpi8 antibodies.**

The transamidase mechanism of GPI anchoring was studied in bloodstream forms of *Trypanosoma brucei* using media containing hydrazine or biotinylated hydrazine. In the presence of the latter nucleophile, part of the newly formed VSG was linked to this instead of the GPI anchor and was not transferred to the cell surface. VSG-hydrazine-biotin was detected by streptavidin in western blots and intracellularly in Golgi-like compartments.

Key words: *Trypanosoma brucei*, Transamidase, GPI membrane anchor, GPI8, Intracellular localization, Variant surface glycoprotein

Introduction

The surface coat of the bloodstream forms of African trypanosomes consists of about 10^7 copies of a variant surface glycoprotein (VSG), which is arranged as a dense monolayer of homodimers on the parasite surface. The ability of trypanosomes to express different variants of VSG, that is, to undergo continuous antigenic variation, enables the parasite to escape from the host's immune response (Cross et al., 1998). VSG is produced as a prepro-protein containing a N-terminal signal sequence and a hydrophobic C-terminal domain, which is post-translationally replaced by a GPI membrane anchor. This membrane anchor exchange is thought to be a single-step reaction performed by a transamidase (Udenfriend and Kodukula, 1995). Since VSG is a dominant protein in trypanosomes, accounting for about 10% of the total amount of protein and is constitutively synthesized at a high rate (about 8% of total protein biosynthesis), this parasite seems to be perfectly suited to study GPI anchor attachment.

In earlier studies, expression of prepromini-placental alkaline phosphatase (MiniPLAP) led to about 10% of protein being cleaved correctly but not transferred to the GPI anchor (Maxwell et al., 1995a). On the basis of these results, a transamidase reaction was supposed, using an activated intermediate of the enzyme, with the carbonyl group of the amino acid at the ω-site of the acceptor. This activated intermediate accepted either the nucleophilic amino group of the ethanolamine residue of GPI to form GPI-linked mature mini-PLAP or an abundant nucleophile such as water to yield free mature mini-PLAP. Direct evidence for a transamidase mechanism came from the observation that a microsomal enzyme activity capable of removing the C-terminal GPI anchor signal was enhanced by small nucleophilic amines (Maxwell et al., 1995b). Here the addition of hydrazine to microsomal membranes led to mature miniPLAP that lacked both the C terminal pro-peptide and GPI. Using a biotinylated derivative of hydrazine instead of hydrazine itself, the released VSG could be precipitated with streptavidin-agarose, indicating that the biotin moiety was covalently linked to the protein (Sharma et al., 1999). In trypanosomes, the reaction was inhibited by sulfhydryl alkylating reagents, suggesting that the transamidase contains a functionally important sulfhydryl residue (Mayor et al., 1991). Transamidase-deficient cells are expected to accumulate complete GPI lipids as well as precursor proteins. This phenotype is exhibited by the 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. It encodes a putative type I transmembrane ER protein with a large luminal domain and shows 27.5% identity to jack bean asparaginyl endopeptidase (Abe et al., 1993). Homologies between this family and other cysteine proteinases, such as caspases, pointed to C199 and H157 being potential active site residues in the yeast protein. Indeed, Gpi8 alleles mutated at C199 or H157 were nonfunctional, that is, they were unable to suppress the lethality of *gpi8* mutants (Meyer et al., 2000). The observed homology with proteases suggests that Gpi8 is directly involved in the proteolytic removal of the GPI-anchoring

signal. In contrast to Gaa1, Gpi8 does not contain any known ER retrieval sequences such as KKXX or KXKXX.

The yeast *GAA*1 gene encodes a 68 kDa protein containing a cytosolic ER retrieval signal at the C-terminus, several membrane spanning domains and a large luminal domain (Hamburger et al., 1995). As shown in the same work, a *gaa1* mutant was defective in the post-translational attachment of GPI to proteins. Thus Gaa1 seems to be required for the attachment of GPI to proteins. Since Gaa1 and Gpi8 co-precipitate, it seems likely that both enzymes together constitute the functional transamidase (Ohishi et al., 2000). More recently, two new components have been reported: PIG-S and PIG-T (Ohishi et al., 2001). They form a complex with Gaa1 and Gpi8, and PIG-S and PIG-T knockout cells were defective in the transfer of GPI to proteins. Gpi16 (Yhr188c) and Gpi17 (Ydr434w) are the orthologues of PIG-T and PIG-S in yeast. Gpi16 is an essential N-glycosylated transmembrane protein, and its depletion results in the accumulation of the complete GPI lipid CP2 and of unprocessed GPI precursor proteins. Gpi8 and Gpi16p are unstable if either one of them is depleted (Fraering et al., 2001).

In the work presented here, *GPI8* from *Trypanosoma brucei* (*TbGPI8*) was cloned, and the recombinant protein (*Tb*Gpi8) was heterologously expressed in *E. coli* and used to generate specific antibodies in chicken. A readout assay was designed to document the in vivo activity of TbGpi8; briefly, *T. brucei* bloodforms were cultured in the presence of tunicamycin and exposed to biotinylated hydrazine to monitor processing of VSG. In addition, activity of the isolated enzyme was demonstrated in vitro using a synthetic substrate Acetyl-S-V-L-N-7-amino-4-methyl-coumarine (Ac-S-V-L-N-AMC), which yields a fluorogenic chromophor after cleavage, and the reaction mechanism was studied using small nucleophilic amines. Localization studies of *Tb*Gpi8 were performed using specific antibodies.

Materials and Methods

Chemicals

Hydrazine, tunicamycin, biotin-LC-hydrazine and isopropylthiogalactoside (IPTG) were from Sigma (Deisenhofen, Germany) and Pierce (Illinois, USA). Anti-CRD antibodies were a gift from T. Ilg (Akzo Nobel, Intervet GmbH, Schwabenheim, Germany). Streptavidin HRP and amylose resin were from Amersham (Braunschweig, Germany). Enterokinase was from Roche (Mannheim, Germany). The fluorogenic peptide substrate (Ac-S-V-L-N-AMC) used for the enzyme assay and all first antibodies were made in our laboratory. Second antibodies either fluoresceineisothiocyanate (FITC) or enzyme labeled were from Sigma (Deisenhofen, Germany). Primers were synthesized by GIBCO BRL (Karlsruhe, Germany).

Cell culture

Bloodstream-form trypanosomes were cultivated at 37°C in a water-saturated atmosphere containing 5% CO_2. Cells were harvested at a density of about 1-2×10^6 ml^{-1}. For glycosylation inhibition and anchor exchange, either 10 μg ml^{-1} tunicamycin and/or 0.5 mM hydrazine was added to culture media overnight unless stated otherwise. Culture medium was minimum essential medium modified by Duszenko et al. (Duszenko et al., 1992).

Preparation of trypanosomal lysates

Freshly isolated trypanosomes (7×10^9 per ml) were lysed in ice-cold phosphate buffer (10 mM, pH 7.4) containing 2 mM DTT, 10 mM 2-aminopurine and 1 μM each of the protease inhibitors pepstatin, leupeptine and chymostatin and immediately homogenized with a Dounce homogenizer. Cell lysis was controlled by phase contrast microscopy and stopped by addition of a 10-fold concentrated isotonic phosphate buffer (180 mM, pH 7.4) to one tenth of the volume after more than 90% of cells were broken. The lysate was centrifuged at 12,000 ***g*** for 6 minutes at 4°C to remove cell debris, mitochondrial membranes and nuclei. The remaining lysate was immediately divided into aliquots and stored in liquid nitrogen.

SDS/PAGE and western blotting analysis

For SDS-polyacrylamide gels a standard protocol (Laemmli, 1970) using 10% running and 5% stacking gels was applied. Western blotting was performed using a Semi-Dry Apparatus (Amersham, Braunschweig, Germany). Three filter papers were placed on the anode plate, before the nitrocellulose membrane, the gel and three additional filter papers were added. All the materials were soaked in transfer buffer (48 mM Tris, 39 mM Glycin, 0.0038% SDS, 20% Methanol, pH 9.2) before use. The electro transfer was carried out by a constant electric current of 5.5 mA cm^{-2} for 30 minutes. Biotin tag, VSG and sVSG were examined by using streptavidin and antibodies specific for VSG and CRD. Gpi8 was detected using anti-Gpi8 antibodies from chicken; control staining was performed using IgY prepared from eggs of pre-immune chicken.

Immunocytochemistry of cells

1×10^7 trypanosomes were fixed in 1 ml formaldehyde in HEPES buffered saline (2.5% formaldehyde, 0.1% glutaraldehyde, 0.85% NaCl, 25 mM HEPES, pH 7.3) for at least 1 hour. Cells were washed twice with cold HEPES buffered saline and cold 1% BSA and resuspended in 400 μl 1% BSA. Cells were permeabilized using 0.1% Triton X-100 and finally resuspended in 0.5 ml PBS containing 1% BSA. 5 μl of this suspension was transferred to glass slides and dried at 37°C for 3 hours or at room temperature overnight. The slide with fixed cells was incubated for 15 minutes with 10 μl of the first antibody in a wet box, washed for 5 minutes with distilled water and dried at room temperature. It was then incubated for 15 minutes with FITC- or TRITC-labeled second antibody and washed again for 5 minutes. For counterstaining of the DNA, the slide was incubated with bisbenzimide for 15 minutes and again dried at room temperature. Slides were viewed and analyzed using an Olympus BH2 fluorescent microscope and imaging software (Soft Imaging System GmbH, Stuttgart, Germany).

Synthesis of Ac-S-V-L-N-AMC

Commercially available 7-amino-4-methylcoumarin (AMC) was coupled with Fmoc-Asn(Trt)-OH using 1 equivalent of isobutylchloroformate in the presence of 1 equivalent of N-ethyldiisopropylamine for 1 hour at –10°C and at room temperature overnight thereafter. Following removal of the solvent (DMF), the reaction mixture was dissolved in ethylacetate and washed three times each with citric acid (2 M) and water. The product was dried over sodium sulfate and precipitated by addition of petrolether. The Fmoc group was removed using 20% piperidine in DMF and stirring for 30 minutes. Acetyl-Ser(tBu)-OH was synthesized by treatment of H-Ser(tBu)-OH with Ac_2O/pyridine in dichloromethane. Boc-Val-Leu-OH was synthesized using Boc-Val-hydroxysuccinimid ester and leucine sodium salt in distilled water/DMF overnight at room temperature. Removal of the Boc protection group was achieved by stirring the reaction mixture for 1 hour at room temperature in TFA:dichloromethane (1:1). The product H-Val-Leu-OH (TFA salt)

was coupled with Ac-Ser(tBu)-hydroxysuccinimidester in a mixture of DMF and water. Finally, Ac-Ser(tBu)-Val-Leu-Asn(Trt)-AMC was produced by a mixed anhydrid coupling reaction of Ac-Ser(tBu)-Val-Leu-OH and Asn(Trt)-AMC in the presence of 1 equivalent of diisopropylethylamine and 1 equivalent of isobutylchloroformate as described above. The remaining protecting groups were removed by addition of TFA for 1 hour at room temperature. The final product was purified by semi-preparative HPLC using a TFA:acetonitril gradient on a hydrophobic C18 column (Nucleosil 300, 5 μm; Machery and Nagel, Düren, Germany). The product was pure as judged by analytical HPLC, and the structure was confirmed using Maldi mass spectroscopy.

Enzyme assay using Ac-S-V-L-N-AMC

Ac-S-V-L-N-AMC (1 mM) was incubated with trypanosomal lysates (10% in 50 mM citrate buffer, pH 5.5) at 30°C. To determine the pH optimum, the reaction mixture was incubated overnight at 30°C in 50 mM citrate buffer at pH values ranging from 2.5 to 6.5 or in 50 mM HEPES buffer at pH values ranging from 5.5 to 9.5. Inhibition studies using sulfhydryl alkylating reagent were performed in the presence of 1 mM pCMPSA at pH 5.5. All assays were performed in the presence of protease inhibitors pepstatin, chymostatin and leupeptin (1 μM each).

Cloning of TbGPI8

Degenerated oligonucleotide primers were designed against highly conserved regions on the basis of the sequences of the yeast and human genes by using BLASTp algorithms (www.ncbi.nlm.nih.gov) (Altschul et al., 1997) and DNA sequences of *T. brucei* obtained by TIGR (TIGR–Databases; www.tigr.org). The 5′-sequence of *TbGPI8* was amplified with Red Taq polymerase (Sigma, Deisenhofen, Germany) using primers GPI8AS1 (CCACATCATCATIA-GIGTITCIGCIATITC)/SLSE23 (CGCTATTATTAGAACAGTTT-CTG) and cloned into pBS KS^+ (Stratagene, The Netherlands). The 3′-sequence was amplified using primers GPI8SE3 (GGACTCGGAGTTCATGAGCTC)/OT203N (CCCGGGT$_{20}$VNN) and cloned into pBS KS^+. cDNA from the bloodstream form MITat 1.2 was used as template. After sequencing by GATC (Konstanz, Germany), two specific primers GPI8SE1 (CGCAGAGGTTTCA-AACAAGTGG)/GPI8AS2 (CTTTGTTGCACGTGACTACAATA) were used to amplify the *TbGPI8*-ORF with Pfu polymerase (Stratagene, The Netherlands) from bloodstream form MIT1.2 cDNA and cloned into pBS KS^+.

Expression of TbGpi8

To clone TbGPI8 into expression vectors, the plasmid pBS KS+ containing TbGPI8 cDNA was used as the template. Plasmid pMAL-c2E (NEB, Schwalbach, Germany) was used for the maltose-binding protein (MBP) fusion system to heterologously express TbGpi8. TbGPI8 was amplified using primers GPSEMAL1 (GCAGCAGGTACCGGCGGAAGGCTTTCATGGTATG)/GPASMA-L2 (GATATAGGTACCCTAGAACAAATCGTAACGTAACTCTAC), cut by KpnI (underlined) and ligated into the KpnI site of pMAL-c2E. Thus TbGpi8 (which is devoid of the putative N-terminal signal sequence) was placed at the C-terminus of MBP.

E. coli strain ER2566 was used to express the fusion protein MBP-*TbGPI8*. When the OD_{600} of the culture reached 0.5-0.8, protein expression was induced at 15°C overnight using 0.3 mM IPTG. Cells were spun down (5000 ***g*** for 10 minutes at 4°C), and cell pellets were stored at –20°C. After thawing, pellets from a 50 ml culture were resuspended in 5 ml ice-cold HEPES buffer (20 mM HEPES, 500 mM NaCl, 1 mM EDTA, pH 7.4) and disrupted by sonification in ice water. A clear cell extract was obtained by centrifugation at 12,000 ***g*** for 30 minutes.

An amylose resin column (2 ml) was equilibrated with 16 ml HEPES buffer, before 5 ml lysate was loaded onto the column and washed with 24 ml HEPES buffer. The fusion protein was eluted with HEPES buffer containing 10 mM maltose. The eluted material was dialyzed against 50 mM Tris-HCl (pH 8.0) and treated with enterokinase (20 μg MBP-TbGpi8 per 1 μg enterokinase) overnight at room temperature.

In vitro activity assay of TbGpi8

Trypanosomal lysates or recombinant *Tb*Gpi8 was mixed with Ac-S-V-L-N-AMC and 10 mM hydrazine. The mixture was incubated at 30°C for 1 hour or overnight and analyzed by spectrofluorometry. (Excitation wavelength: 360 nm; emission scan: 370-530 nm.)

Anti-TbGpi8 IgY

A chicken was immunized with isolated MBP-*Tb*Gpi8. After the first immunization using 0.5 mg isolated protein and Freunds complete adjuvants, the chicken was boostered twice with 0.25 mg protein in Freunds incomplete adjuvants on day 28 and day 42 after the initial immunization. The increase of antibody titer was controlled by ELISA tests.

Antibodies were prepared according to Polson et al. (Polson et al., 1980) and Gassmann et al. (Gassmann et al., 1990). Briefly, three egg yolks were carefully separated from egg whites, mixed with 25 ml phosphate buffer (1.8 g/l Na_2HPO_4, 1.4 g/l KH_2PO_4, 5.8 g/l NaCl, pH 7.2) and 75 ml PEG (7%) and incubated at 4°C for 30 minutes. After centrifugation at 3000 ***g*** for 10 minutes at 4°C, the supernatant was filtered through mull. PEG was added to the filtrate to a final concentration of 12% and incubated at 4°C for 30 minutes. After centrifugation at 3000 ***g*** for 10 minutes at 4°C, the pellet was resuspended in 50 ml phosphate buffer and 50 ml 24% PEG and incubated at 4°C for 30 minutes. The mixture was centrifuged at 3000 ***g*** for 10 minutes 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 minutes at 4°C the pellet was dissolved in 10 ml phosphate buffer and centrifuged at 3000 ***g*** for 10 minutes at 4°C again. The supernatant was stored at –20°C.

10 ml isolated chicken antibodies were run through a 1 ml amylose column containing MBP. The flow-through without antibodies 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, and the flow-through was discarded. Specific antibodies against *Tb*Gpi8 were eluted with 100 mM glycine/HCl at pH 2.5 thereafter.

Results

Cloning of TbGPI8

We have cloned and sequenced *GPI8* from *T. brucei* using cDNA from bloodstream forms as the template. *TbGPI8*-ORF is 960 bp long, including the stop codon TAG and codes for a polypeptide of 319 amino acids. Sequence analysis revealed one hydrophobic region at the N terminus, which by homology qualifies as a signal peptide for translocation into the endoplasmic reticulum (von Heijne, 1986). As calculated from the amino acid sequence, *Tb*Gpi8 has a pI of 5.51 and a molecular mass of 37 kDa or 35 kDa if the signal sequence is removed. The cleavage site for the signal peptidase is expected between residues A17 and A18. By comparison with the respective proteins from other eukaryotic organisms, *Tb*Gpi8 shows a high degree of homology (Fig. 1a). Sequence alignment also indicates that C192 and H150 of *Tb*Gpi8 represent the conserved active site residues, which are located at position

C199 and H157 in *Sc*Gpi8 (Meyer et al., 2000). Unlike *Sc*Gpi8, *Tb*Gpi8 has no hydrophobic membrane-spanning region at the C-terminus and is thought to be a soluble protein (Fig. 1b).

Expression and purification of TbGpi8 in *E. coli*

The MBP fusion protein expression system was used to express *Tb*Gpi8. *E. coli* strain 2566 containing *TbGPI8* in plasmid pMAL-c2E was induced overnight with 0.3 mM IPTG at 15°C. Cells were sonicated using short pulses of 15 seconds, each for about 15 minutes, then centrifuged. The supernatant was applied onto an amylose resin column and purified. Following this affinity chromatography, the MBP-*Tb*Gpi8 fusion protein showed a single band on SDS-PAGE with an apparent mass of about 80 kDa (Fig. 2). In this way, about 2 mg of MBP-*Tb*Gpi8 was isolated from a 50 ml culture. To obtain pure *Tb*Gpi8, enterokinase cleavage was carried out using a ratio of 1:50 (w/w) cleaving enzyme to fusion protein. The reaction mixture was incubated overnight at room temperature and analysed by SDS-PAGE. As shown in Fig. 3, several protein bands appeared in addition to *Tb*Gpi8, MBP and enterokinase, which were removed, however, by using an amylose resin column. To avoid confusion with MBP (molecular weight: 42 kDa), the flow-through was run again over an amylose column, and the flow-through of the second column was analysed by SDS/PAGE (Fig. 3, lane 2). Although the apparent molecular mass was higher (about 48 kDa) than calculated from the amino acid sequence (35 kDa), we assume that this protein is mature *Tb*Gpi8 because it contains no cleavage site for enterokinase. All MBP-linked products were absent from the flow-though of the amylose resin column but were easily eluted with maltose (Fig. 3, lane 3); enterokinase light chain, as used in our experiments, has a molecular weight of 26.5 kDa and runs at an apparent molecular weight of 31 kDa on a SDS-PAGE (data obtained from the manufacturer). We contribute the observed differences in the apparent molecular weight of the recombinant *Tb*Gpi8 to a slightly different folding pattern, rather then to a remaining part of MBP.

In vitro peptidase assay using an AMC-linked peptide as substrate

The use of 7-amino-4-methyl-coumarin (AMC) fluorogenic peptide substrates is a well established method for the determination of protease activity and specificity (Zimmerman et al., 1977). The substrate is stable in solution and closely related to 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 GPI attachment (ω-site) in VSG is, as far as is known so far,

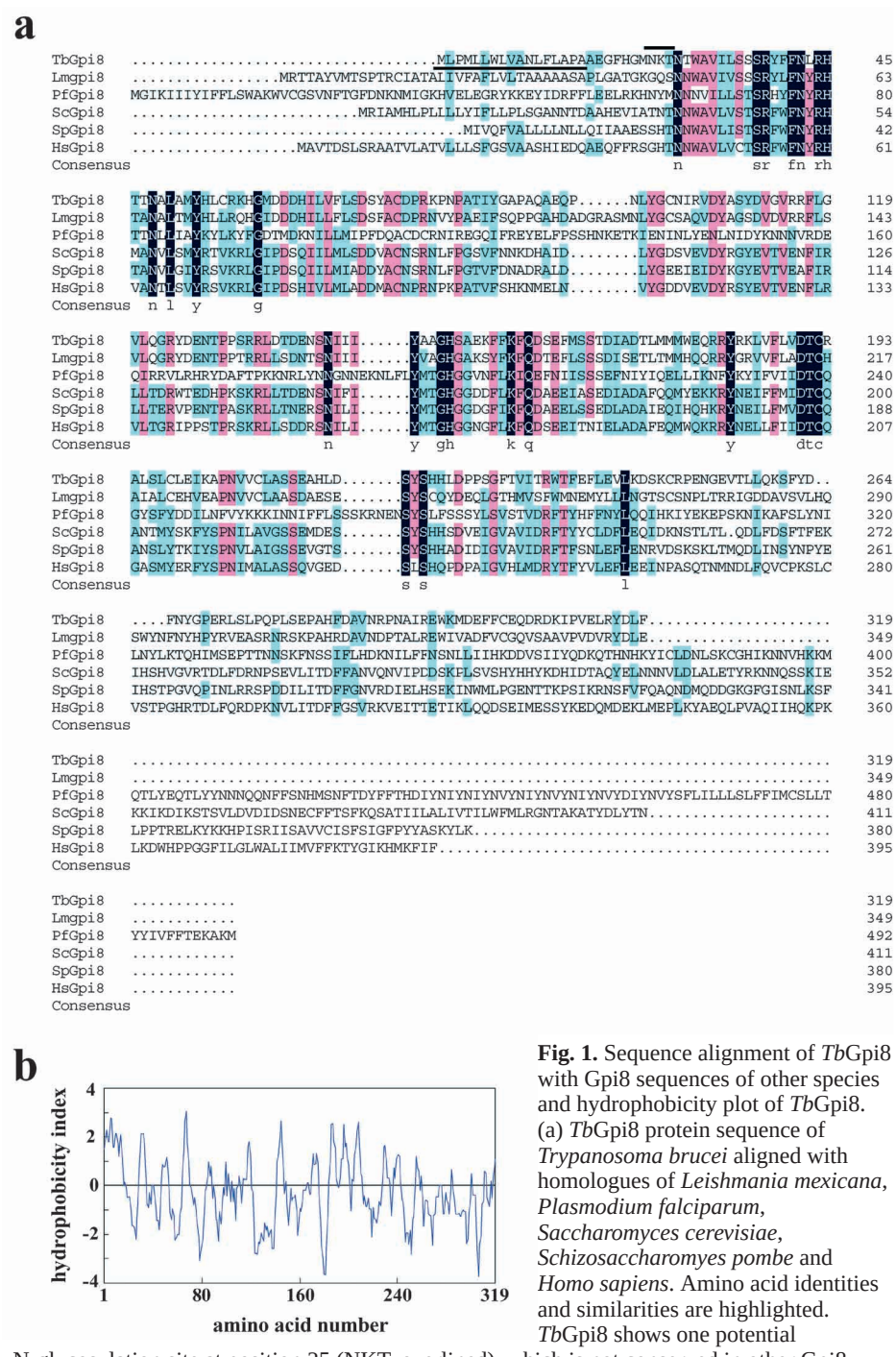

Fig. 1. Sequence alignment of *Tb*Gpi8 with Gpi8 sequences of other species and hydrophobicity plot of *Tb*Gpi8. (a) *Tb*Gpi8 protein sequence of *Trypanosoma brucei* aligned with homologues of *Leishmania mexicana*, *Plasmodium falciparum*, *Saccharomyces cerevisiae*, *Schizosaccharomyes pombe* and *Homo sapiens*. Amino acid identities and similarities are highlighted. *Tb*Gpi8 shows one potential N-glycosylation site at position 25 (NKT, overlined), which is not conserved in other Gpi8 molecules. In addition, *Tb*Gpi8 has a putative hydrophobic N-terminal ER-directing signal sequence (underlined), but shows no hydrophobic membrane-spanning region at its C-terminus when compared with other Gpi8 molecules. (b) Hydrophobicity plot of TbGpi8 using a window of 6 amino acids: note that there is no putative transmembrane domain on the C-terminus of the protein.

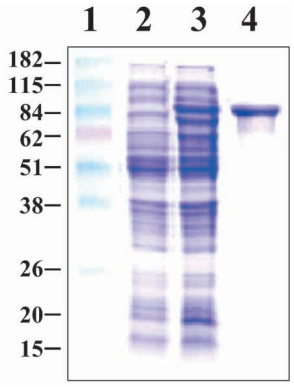

Fig. 2. Induction and purification of MBP-*Tb*Gpi8. Cells were induced using 0.3 mM IPTG and lysed by sonification. MBP-*Tb*Gpi8 was eluted with elution buffer containing 10 mM maltose and analysed by SDS-PAGE. Lane 1, molecular weight markers; lane 2, lysate from non-induced cells; lane 3, cells induced with 0.3 mM IPTG; lane 4, MBP-*Tb*Gpi8 after purification using an amylose column.

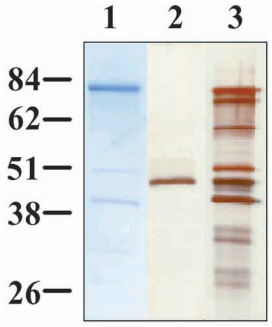

Fig. 3. Enterokinase treatment of MBP-*Tb*Gpi8. Enterokinase cleavage was carried out using an enzyme:MBP-*Tb*Gpi8 ratio of 1:50 (w/w) in 50 mM Tris/HCl buffer at pH 8.0 and room temperature for 8 hours. The cleavage mixture was run through an amylose column to remove MBP or MBP-linked proteins. Samples were analysed by SDS/PAGE. Lane 1, purified MBP-*Tb*Gpi8 after the first run of amylose column (Coomassie staining); lane 2, flow-through of the amylose column after enterokinase treatment of the fusion protein; lane 3, proteins eluted from the amylose column using10 mM maltose; lanes 2 and 3, silver staining.

one of the following amino acids: D, S or N (Cross, 1990). We used the synthetic peptide Ac-S-V-L-N-AMC as a substrate to test for a transamidase activity in trypanosomal lysates. The excitation and emission maxima of amino-acid-conjugated AMC substrates are 350 nm and 400 nm, respectively. Cleavage of the substrate will release the free AMC residue and results in a shift of the excitation and emission maxima to 340 nm and 440 nm, respectively. Hydrolysis of the substrate was monitored fluorometrically with an excitation wavelength of 350 nm and an emission scan from 370 nm to 530 nm using a spectrofluorimeter. Ac-S-V-L-N-AMC was incubated in the presence of 1 μM protease inhibitors (leupeptin, pepstatin and chymostatin) at 30°C for 1 hour using 1 mM substrate in 50 mM citrate buffer at pH 5.5. As shown in Fig. 4a, the emission maxima changed from 400 nm to about 440 nm after addition of trypanosomal lysates, suggesting that the lysates contained an active enzyme, which catalyzed the cleavage of the anilide bond. Using trypanosomal lysates adjusted to different pH values, the highest emission was seen at pH 5.5, whereas a rapid decrease in activity was measured at more acidic or basic condition: at pH 4.5 or pH 6.5 only about 10% of the enzyme activity remained (Fig. 4b).

To further analyze whether a transamidase reaction is involved in Ac-S-V-L-N-AMC cleavage, we used hydrazine and *p*-chloro-mercuriphenyl-sulfonic acid (pCMPSA). A nucleophilic attack by GPI, water or a nucleophile such as hydrazine is the final step of the transamidation reaction (Udenfriend and Kodukula, 1995). In lysates containing 10 mM hydrazine, a significantly higher emission was observed at λ=440 nm than in samples without hydrazine (Fig. 4c). As shown previously, the GPI anchoring reaction in bloodstream-stage trypanosomes is inhibited by the sulfhydryl alkylating reagent pCMPSA (Mayor et al., 1991; Sharma et al., 2000), suggesting that a histidine and/or a cysteine residue is located at the active site (Meyer et al., 2000). We have checked the effect of pCMPSA as well, and we found a strong inhibition of AMC release (Fig. 4d).

At least two proteins participate in the transamidation reaction; these include Gaa1 and Gpi8. Therefore, mutant yeast

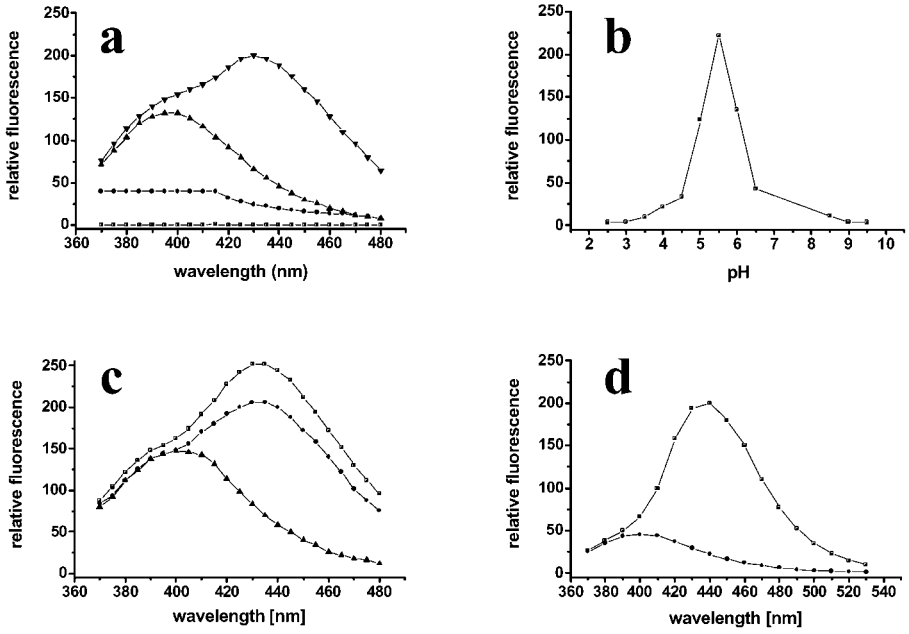

Fig. 4. Enzyme activity in trypanosomal lysates to cleave Acetyl-SVLN-AMC. 1 mM substrate was incubated with trypanosomal cell lysates (10% in citrate buffer, pH 5.5) at 30°C. After incubation, the shifts of emission maxima were measured. Lysate and buffer shifts were also measured at mock condition. For determination of the pH optimum (b), samples were incubated 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 in the Materials and Methods. (a) Addition of trypanosomal lysate led to the release of AMC from the peptide substrate (▼), while substrate alone (▲), lysate alone (●) or buffer alone (■) did not. (b) Determination of the pH optimum. (c) Effect of hydrazine addition on enzyme activity: liberation of AMC increased significantly after addition of 10 mM hydrazine (■) as compared with experiments without hydrazine (●). Again, substrate alone was stable. (d) Effect of pCMPSA addition on enzyme activity: trypanosome lysates and substrate were incubated in the presence (●) or absence (■) of 1 mM pCMPSA as described in the Materials and Methods.

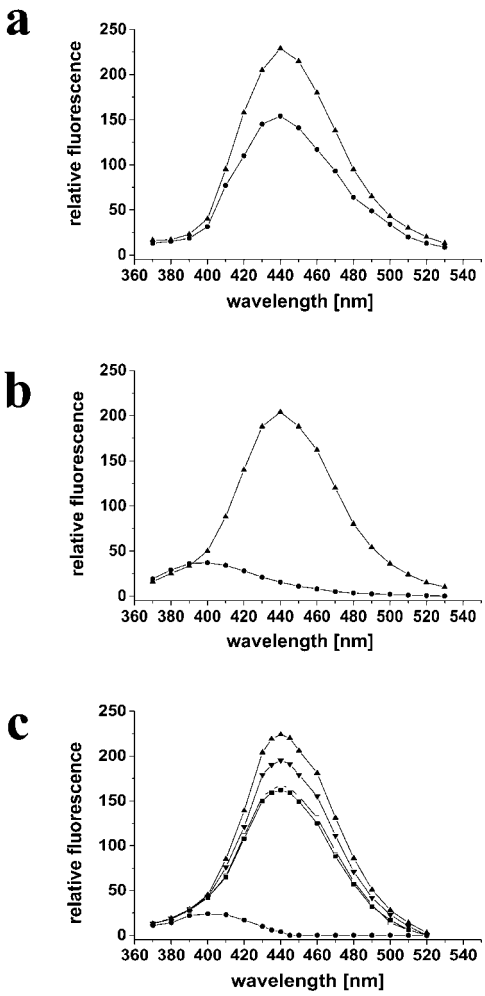

Fig. 5. Transamidase activity of *Tb*Gpi8 and MBP-*Tb*Gpi8. (a) Trypanosomal lysate and substrate were incubated at 30°C in the presence (▲) or absence (●) of recombinant *Tb*Gpi8. (b) Recombinant *Tb*Gpi8 (▲) or MBP-*Tb*Gpi8 (●) and substrate were incubated at 30°C. (c) Trypanosomal lysates were preincubated with Gpi8-specific antibodies for 1 hour at room temperature before the enzyme assay was performed. (▲) control assay without antibodies; (▼) 60 pg/ml antibody; (□) 120 μg/ml antibody, (■) 240 pg/ml antibody, (●) substrate alone.

cells deficient in either Gaa1 or Gpi8 failed to express GPI-anchored proteins (Benghezal et al., 1996; Hamburger et al., 1995). Assuming that trypanosome lysates contain the active transamidase complex, addition of recombinant *Tb*Gpi8 should increase the endogenous Gpi8 concentration and thus enhance the activity of the enzyme complex (Sharma et al., 2000). To check for this possibility, isolated *Tb*Gpi8 was incubated together with trypanosome lysate and the synthetic peptide substrate Ac-S-V-L-N-AMC overnight at 30°C in citrate buffer containing 10 mM hydrazine (see below) and the protease inhibitors leupeptin, pepstatin and chymostatin (1 μM each) at pH 5.5. Under these conditions, the cleavage capacity was significantly elevated by the recombinant protein compared with the lysate alone (Fig. 5a).

Since Gpi8 bears resemblance to several plant and invertebrate proteases, it could have some proteolytic activity itself. On the basis of this assumption, the transamidase activity

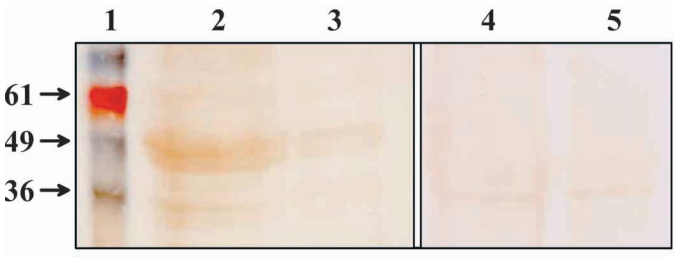

Fig. 6. Western blot detecting *Tb*Gpi8 with specific IgY antibodies. Trypanosomal lysate was separated by SDS-PAGE and analysed for *Tb*Gpi8 using affinity-purified chicken anti-*Tb*Gpi8 antibodies (lanes 2 and 3) or unspecific IgY antibodies isolated from non-immunized eggs (lanes 4 and 5). Lane 1, molecular weight standards, lanes 2 and 4, lysates equivalent to 1×10^7 cells per lane; lane 3 and 5, lysates equivalent to 5×10^6 cells per lane.

of *Tb*Gpi8 was tested in the absence of trypanosomal lysates. As before, when Ac-S-V-L-N-AMC was incubated in the presence of recombinant *Tb*Gpi8, the AMC residue was cleaved off, as shown by a significant increase in light absorption at λ=440 nm. Interestingly, AMC was produced by the isolated *Tb*Gpi8 but not by MBP-*Tb*Gpi8 (Fig. 5b). These results indicate that recombinant *Tbg*Gpi8 has a peptidase activity itself and cleaves Acetyl-S-V-L-N-AMC at the P1 position, which corresponds to the ω position in VSG, leading to the removal of the C-terminal peptide. When enterokinase was used instead of the isolated *Tb*Gpi8, no release of AMC was monitored at the enterokinase cleavage site, which is at K position in the DDDDK sequence (data not shown). In addition, trypanosome lysates were incubated in the presence or absence of Gpi8-specific antibodies to monitor whether the enzyme is inhibited. As shown in Fig. 5c, enzyme activity is reduced to a maximum of about 60%, but not completely blocked. We assume that binding of the polyclonal antibody is not in the area of the active centre.

Intracellular localization of TbGpi8 using isolated anti-TbGpi8 IgY

For technical reasons, MBP-*Tb*Gpi8 was used to produce antibodies by immunisation of a hen. IgY was isolated from egg yolk by PEG precipitation. Specific anti-*Tb*Gpi8 IgY was affinity purified using a MBP-amylose column. The flow-through was applied to a MBP-*Tb*Gpi8-amylose column, and bound antibodies were eluted at pH 2.5. The specific *Tb*Gpi8 antibodies isolated in this way were used for western blotting and immunolocalization studies. Using affinity-purified anti-*Tb*Gpi8 IgY, the protein was detected by SDS-PAGE and western blotting. As shown in Fig. 6, the antibodies specifically detected two bands showing apparent masses of 48 kDa and 45 kDa in trypanosomal lysates. Since the apparent molecular mass calculated from the protein sequence is 35 kDa, then Gpi8 must run, for unknown reasons, at a higher molecular mass by the SDS-PAGE (compare with Fig. 3). The Gpi8 sequence shows one putative N-glycosylation site. Thus the 45 kDa may represent either a non glycosylated protein or a protein non-specifically labeled by our antibody.

For intracellular immunolocalization of *Tb*Gpi8, formaldehyde fixed cells were labeled with anti-*Tb*Gpi8 IgY, whereas the nucleus and kinetoplast were counterstained using

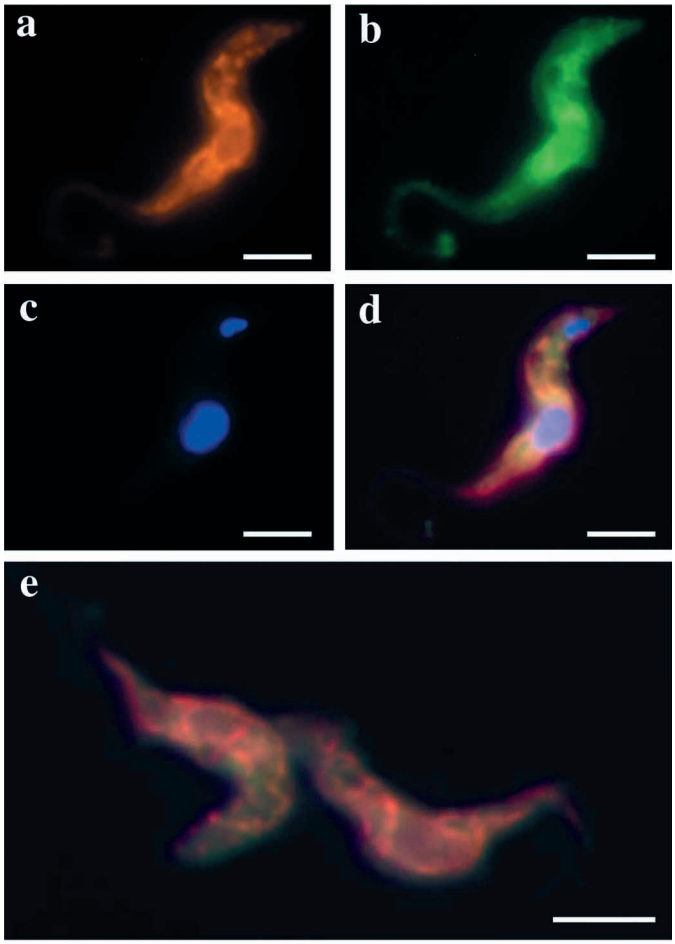

Fig. 7. Intracellular localization of TbGpi8 in bloodstream-form trypanosomes. Affinity-purified chicken anti-*Tb*Gpi8 antibodies have been used to localize TbGpi8 in intracellular compartments. This labelling was localized specifically around the nucleus and in tubular and vesicular structures throughout the cell. (a-d), trypanosomes were sequentially stained with anti-Gpi8 antibodies and FITC-labelled anti-chicken IgG (a), anti-BiP antibodies and TRITC-labelled anti-rabbit IgG (b) and bisbenzamide (c). (d) shows an overlay of all images. (e) overlay of Gpi8- and BiP-stained cells. Bars, 5 μm.

bisbenzimide. The results are shown in Fig. 7. Labelling around the nucleus and in tubular and vesicular structures throughout the cell was visible. Colocalization studies using BiP-specific antibodies, kindly provided by J. D. Bangs (Wisconsin University), strongly indicates an ER-specific localization.

Anchor exchange mechanism in trypanosomes

Nonglycosylated VSG

Tunicamycin is a hydrophobic analogue of UDP-N-acetylglucosamine. It blocks addition of N-acetylglucosamine to dolicholphosphate, that is, the first step to form N-linked oligosaccharides. In order to produce non-glycosylated VSG with a molecular weight that differs from that of the already processed 'old' VSG, cells were cultivated in the presence of

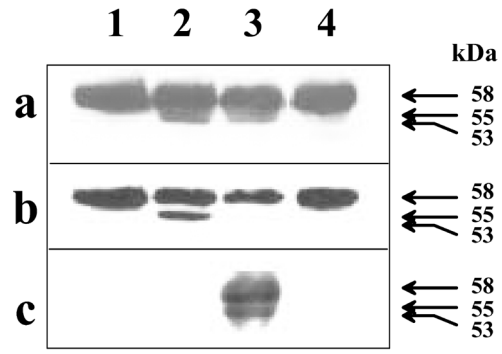

Fig. 8. Effect of tunicamycin and hydrazine or hydrazine-biotin on VSG formation. Trypanosomes were cultivated in the presence of 10 μg ml^{-1} tunicamycin (lanes 2, 3 and 4) and 10 mM hydrazine (lane 4) or 10 mM hydrazine-biotin (lane 3) for 4 hours at 37°C in a CO_2 incubator. Following cultivation, cells were analysed by SDS-PAGE and western blotting using anti-VSG MITat 1.2 specific antibodies (a), anti-CRD specific antibodies (b) or streptavidin-HRP (c). Lane 1 shows the control sample incubated in the absence of both tunicamycin and hydrazine.

10 μg ml^{-1} tunicamycin. After 4 hours in culture and following separation of proteins by SDS-PAGE, two additional VSG bands with distinctively lower molecular weights from the mature VSG were detected on western blots using anti-VSG antibodies (Fig. 8). Since glycosylation of VSG was efficiently inhibited by tunicamycin, we used this method throughout the following experiments. In order to detect different forms of VSG, we used either trypanosome clone-specific antibodies (to detect the VSG antigen type), CRD-specific antibodies [to detect the cross-reacting determinant of the GPI anchor (Zamze et al., 1988)] or streptavidin HRP (to detect biotinylated VSG). In this way, the various VSG forms could be distinguished (Table 1).

Use of hydrazine and hydrazine-biotin as substitutes for GPI during the transamidation reaction

The proposed mechanism of GPI transfer is a transamidation reaction that involves formation of an activated carbonyl intermediate (enzyme-substrate complex) with the ethanolamine moiety of the pre-assembled GPI unit serving as a nucleophile. Hydrazine and hydroxylamine are well known nucleophilic acceptors in transpeptidase (Tate and Meister, 1974a; Tate and Meister, 1974b) and transamidase (Buchanan, 1973) reactions. They have also been shown to serve as alternative substrates for GPI using engineered protein miniplacental alkaline phosphatase (promini-PLAP) and rough microsomal membranes of HeLa cells (Maxwell et al., 1995b; Ramalingam et al., 1996). According to the suggested mechanism for the GPI anchor exchange, the carbonyl group of the so-called ω amino acid of promini-PLAP (Udenfriend and Kodukula, 1995) or pro-VSG (Sharma et al., 1999) is activated by a sulfhydryl group within the active center of the transamidase, resulting in formation of an enzyme-substrate complex and cleavage of the amide bond between ω and ω + 1. Thus a nucleophilic attack of H_2N-X will result in formation of protein-NH-X and regeneration of the active site sulfhydryl residue. In our case, use of hydrazine or hydrazine-biotin

Table 1. Detection of the various VSG forms

VSG form	Anti-MITat 1.2 Ab	Anti-CRD Ab	Streptavidin-HRP
Mature VSG	+	(+)*	–
Pro VSG	+	–	–
Biotinylated VSG	+	–	+

*Anti CRD antibodies recognize soluble (s) but not membrane-form (mf) VSG. sVSG is formed from mfVSG during lysis by action of the endogenous GPI specific phospholipase C.

should lead to the formation of VSG-NH-NH_2 or VSG-NH-NH-biotin, which both should not be detected by anti-CRD antibodies, owing to the lack of GPI anchor (see Table 1). To discover whether other nucleophiles could compete with the GPI anchor in vivo, trypanosomes were cultivated in the presence of 10 mM hydrazine or 10 mM hydrazine-biotin for 4 hours. After SDS-PAGE and western blotting, the same blot was subsequently analysed using anti-MITat 1.2 antibodies, anti-CRD antibodies and streptavidin-HRP (Fig. 8). If trypanosomes were cultivated in the absence of hydrazine or hydrazine-biotin, non-glycosylated VSG was readily labeled with anti-CRD antibodies, whereas VSG produced in the presence of hydrazine or hydrazine-biotin was not (Fig. 8). Our data show that hydrazine and hydrazine-biotin could enter the cells, function as nucleophiles in the transamidation reaction and form hydrazine derivatives of VSG. All of these results are consistent with the putative anchor exchange mechanism, as mentioned above.

Immunofluorescence of hydrazine-treated trypanosomes

In earlier experiments, hydrazine was used at a concentration of 10 mM (Chen et al., 1996; Sharma et al., 1999). However, since trypanosomes treated with 10 mM hydrazine survived for only 5 to 6 hours under cultivation conditions, the following experiments have been performed in the presence of 0.5 mM hydrazine, which parasites survived in for at least 44 hours. In trypanosomes, the intracellular transport and export of VSG is critically dependent on the presence of the GPI anchor (Bangs et al., 1996; Bangs et al., 1997). In addition, McDowell et al. have also investigated the role of the GPI anchor in forward secretory trafficking using African trypanosomes (McDowell et al., 1998). Here soluble GPI-minus forms of VSG, in which the C-terminal peptide was deleted, were transported with a five-fold reduction in their kinetics, and immunofluorescent localization studies have indicated that the GPI-minus VSG accumulates within the ER. To determine the location of VSG-hydrazine, we performed immunofluorescence assays on cultivated bloodstream forms. After cultivation for 44 hours in the presence of 0.5 mM hydrazine, cells were fixed with formaldehyde and stained with clone-specific antibodies (anti-MITat 1.2 antibodies); counterstaining was performed using bisbenzimide to stain the nucleus and the kinetoplast. As shown in Fig. 9, VSG was equally distributed on the cell surface, whereas vesicles within the cells could not be detected in the absence of hydrazine (panel a). In contrast, in hydrazine-treated cells (panel b) intensively stained vesicles were observed between the nucleus and kinetoplast, whereas cell surface staining was relatively minor. This result is readily explained by the assumption that, consistent with the results obtained by western blotting, hydrazine served as a substitute for GPI, leading to VSG-hydrazine. Obviously, this VSG-hydrazine accumulated within cellular compartments, which stained much brighter than the cell surface, although the surface coat was still intact as judged by electron microscopy (data not shown).

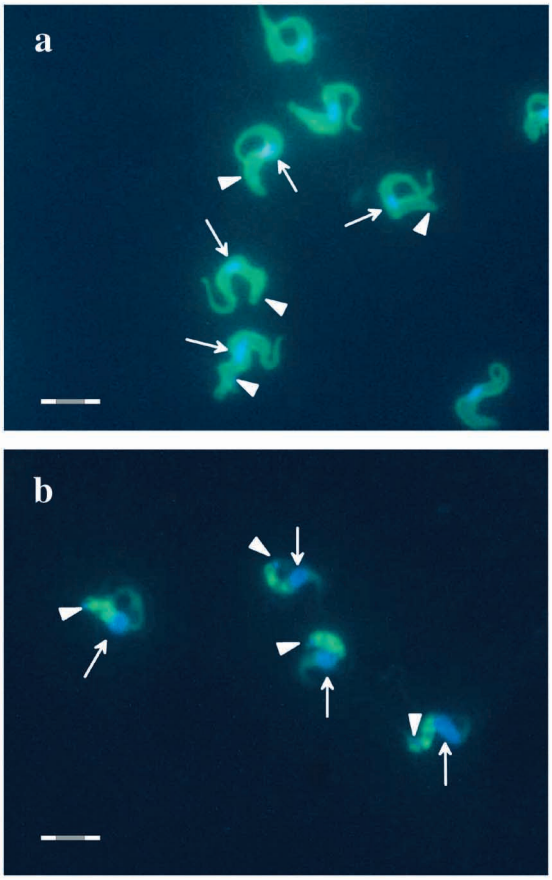

Fig. 9. Intracellular localization of VSG-hydrazine-biotin. Bloodstream-form trypanosomes were grown for 44 hours in the presence (b) or absence (a) of 0.5 mM hydrazine. Parasites were fixed in HBS buffer containing 1% formaldehyde. VSG was immunolocalized using anti-VSG MITat 1.2 specific antibodies. Bars, 5 μm; arrows and arrowheads denote the nucleus and kinetoplast DNA respectively, which were stained by bisbenzimide.

Discussion

GPI-linked membrane proteins are synthesized with a hydrophobic C-terminal sequence containing a signal for their own cleavage. Immediately after cleavage, the C-terminal peptide is replaced by a preformed GPI precursor. Cleavage and replacement is thought to be a transamidation reaction (Udenfriend and Kodukula, 1995) that is dependent on at least two proteins, Gaa1 and Gpi8 (Hamburger et al., 1995), but possibly even more (Vidugiriene et al., 2001). In order to investigate this reaction in trypanosomes, we cultivated the parasites in the presence of 10 ng ml^{-1} tunicamycin to produce non-glycosylated VSG. This VSG runs faster in SDS-PAGE than glycosylated VSG and allows analysis of newly formed

VSG without interference of glycosylated VSG from within the cells. VSG of clone MITat 1.2 contains two N-glycosylation sites. Since tunicamycin did not block N-glycosylation completely, we observed three VSG molecules, one indistinguishable from mature VSG, one containing only one glycan residue and the non-glycosylated form. Using tunicamycin and small nucleophiles at the same time, the experiments clearly showed that the latter substitute for GPI in the anchoring reaction because in the presence of hydrazine or biotin-hydrazine none of the smaller VSG bands could be detected using anti-CRD antibodies, whereas in the presence of biotin-hydrazine all biotin-tagged VSG was readily detected by streptavidin.

In some cases, the GPI anchor acts as a signal to target proteins to the apical site of the plasma membrane (Lisanti et al., 1989; Lisanti et al., 1990); but it may also function in the export of proteins from the ER. Normally, VSG is transported along the classical intracellular route for glycoproteins and is delivered to the flagellar pocket, where it is integrated into the surface coat (Duszenko et al., 1988). Disruption of GPI attachment, either in cell lines deficient in formation of the GPI precursor or in cell lines mutated in the C-terminal signal sequence, show retention of otherwise GPI-anchored proteins within the ER (Moran et al., 1992; Field et al., 1994). McDowell et al. have investigated the transport of VSG minus GPI (McDowell, 1998). Their studies indicate that GPI-minus VSG accumulates within the ER. This delayed forward transport is not caused by a failure to fold or assemble in the absence of the GPI anchor. Instead, the GPI structure seems to act in a positive manner to mediate efficient forward transport of some, and perhaps all, GPI-anchored proteins in the early secretory pathway of trypanosomes (McDowell et al., 1998). Therefore, in our experiments, if the GPI anchor was replaced by a small nucleophile, similar results were expected. Immunolocalization of VSG showed, however, that VSG-hydrazine accumulates in several extensively stained vesicular structures exclusively located between the nucleus and kinetoplast and do not stay in the tubular structures of ER as suggested from experiments using VSG minus GPI (McDowell et al., 1998). However, VSG minus GPI was synthesized as VSG minus the C-terminus within the ER and not further transported. In our experiments, VSG was synthesized as pro-VSG in the ER containing the hydrophobic C-terminal peptide as a possible membrane anchor before hydrazine was added instead of GPI. Obviously, transport of hydrazine-VSG is different from transport of mature GPI-VSG or VSG minus GPI.

In our experiments, Ac-S-V-L-N-AMC was cleaved in trypanosomal lysates, leading to the release of the fluorogenic AMC residue. About 30 families of peptidases are dependent on a cysteine residue at the active center (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 superfamily. These are predominantly lysosomal enzymes, which do not show substrate specificity for asparaginyl residues but have a preference for bulky hydrophobic residues at the P1 position such as valine and phenylalanine (Harris et al., 2000). In trypanosomal lysates the enzyme reaction was optimal at pH 5.5, the pH optimum of the transamidase reaction, and was activated by 10 mM hydrazine, a well known nucleophilic acceptor in transamidase and trans-peptidase reactions (Tate and Meister, 1974a; Tate and Meister, 1974b). Cleavage activity was completely inhibited by 1 mM pCMPSA, a sulfhydryl alkylating reagent and increased by addition of recombinant *Tb*Gpi8. Although we cannot completely rule out the possibility that other proteases led to the observed cleavage of Ac-S-V-L-N-AMC in trypanosomal lysates, we suppose that this reaction is performed by the trypanosomal transamidase, which has a high homology to other known Gpi8 proteins and C13 cysteine peptidases such as legumain (Chen et al., 1997).

To gain a better understanding of the properties of *Tb*Gpi8, the respective cDNA was cloned, and the protein was heterologously expressed and characterized. As compared with *Gpi8* genes from other species, *TbGpi8* shows a high sequence and size homology to *LmGpi8*. In addition, sequence and hydrophobicity analysis indicated a conventional N-terminal signal sequence for ER translocation and a possible N-glycosylation site on N25, which is close to the most probable cleavage site for the signal peptidase (von Heijne, 1986).

Like *Lm*Gpi8, *Tb*Gpi8 lacks a C-terminal hydrophobic domain, which was found in yeast and human Gpi8 and may serve as a transmembrane helix (Benghezal et al., 1996). However, this transmembrane domain seems not to be necessary for protein function, since a Gpi8 mutant from man, which lacks the transmembrane domain, retained its activity to complement class K mutant cells (Ohishi et al., 2000). Hilley et al. suggested that attachment of *L. mexicana Gpi8* to the ER membrane may require one or more integral membrane proteins to be part of the transamidase complex (Hilley et al., 2000). A possible candidate is Gaa1. The exact role of this protein was not elucidated yet, but it had been demonstrated that Gaa1 and Gpi8 form a protein complex (Ohishi et al., 2000). In human and yeast cells, Gaa1 is a luminal oriented ER glycoprotein containing several transmembrane domains (Hamburger et al., 1995). As shown by Ohishi et al., *GAA1* knockout cells were defective in the formation of carbonyl intermediates between precursor proteins and the transamidase (Ohishi et al., 2000). Using the BLAST program and TIGR-Database we have also found a *TbGAA1* gene that shows a high homology with *hGAA1* and *ScGAA1* and contains several possible transmembrane domains (data not shown). This protein is not yet cloned and needs to be further characterized.

As judged from our experiments, cellular localization using specific antibodies shows that *Tb*Gpi8 is mainly localized within the ER compartment. This was expected, because GPI addition is a quasi co-translational process occurring immediately after VSG translation (Bangs et al., 1985; Ferguson et al., 1986). Although *Tb*Gpi8 contains no ER retrieval motif (KDEL or KKXX), it seems likely that Gpi8 forms an active enzyme complex with Gaa1 and is thus kept within the ER and cis-Golgi complex (Lotti et al., 1999). Interestingly, intracellular staining of *Tb*Gpi8 with specific antibodies was much brighter after cells had been exposed to hydrazine. This observation indicates that *Tb*Gpi8 is induced by increasing amounts of preformed GPI precursor or of non-GPI anchored VSG.

Gpi8 shares significant homology with a family of previously characterized asparaginyl endopeptidases known as legumains (Benghezal et al., 1996; Ishii, 1994). These enzymes have been categorized as the C13 family of cysteine peptidases, which also contains the GPI linked protein transamidase

(Riezman and Conzelmann, 1998). It seems likely that the trypanosomal transamidase contains the classical catalytic dyad residues cysteine and histidine, which mediate its activity. It has been shown that GPI linked protein transamidase activity is susceptible to sulfydryl alkylating agents, implying that the protein has an essential cysteine (Sharma et al., 1999). Our study of *Tb*Gpi8 in trypanosomal lysates also showed an efficient inhibition of the transamidase activity by pCMPSA. Using *Tb*Gpi8 numbering, two cysteines (C76 and C192) and two histidines (H45 and H150) are conserved among trypanosomal, leishmanial, yeast and human Gpi8. Both histidine residues are also conserved in legumains, whereas only one of the cysteine residues is conserved across all C13 family members (C192, *Tb*Gpi8 numbering). This residue is thus the prime candidate for the active site cysteine. Meyer et al. proved that C199 and H157 in *Sc*Gpi8 are the active site residues (Meyer et al., 2000). In *Tb*Gpi8, it is most likely that the two corresponding residues, C192 and H150, constitute the active site. The detected enzyme activity of the recombinant *Tb*Gpi8 suggests that the enzyme is in its mature form, despite its different apparent molecular mass.

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