

Essential Roles for GPI-anchored Proteins in African Trypanosomes Revealed Using Mutants Deficient in GPI8

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The survival of *Trypanosoma brucei*, the causative agent of Sleeping Sickness and Nagana, is facilitated by the expression of a dense surface coat of glycosylphosphatidylinositol (GPI)-anchored proteins in both its mammalian and tsetse fly hosts. We have characterized *T. brucei* GPI8, the gene encoding the catalytic subunit of the GPI:protein transamidase complex that adds preformed GPI anchors onto nascent polypeptides. Deletion of *GPI8* (to give Δ *gpi8*) resulted in the absence of GPI-anchored proteins from the cell surface of procyclic form trypanosomes and accumulation of a pool of non-protein-linked GPI molecules, some of which are surface located. Procyclic Δ *gpi8*, while viable in culture, were unable to establish infections in the tsetse midgut, confirming that GPI-anchored proteins are essential for insect-parasite interactions. Applying specific inducible *GPI8* RNAi with bloodstream form parasites resulted in accumulation of unanchored variant surface glycoprotein and cell death with a defined multinuclear, multikinoplast, and multiflagellar phenotype indicative of a block in cytokinesis. These data show that GPI-anchored proteins are essential for the viability of bloodstream form trypanosomes even in the absence of immune challenge and imply that GPI8 is important for proper cell cycle progression.

INTRODUCTION

Trypanosoma brucei is the heteroxenous, hemoflagellate protozoan parasite responsible for Sleeping Sickness in humans and Nagana in domestic animals in the tsetse belt of sub-Saharan Africa. All life cycle stages of the parasite utilize glycosylphosphatidylinositol (GPI) anchors as the predominant method for attaching proteins to their plasma membrane. In the mammalian host, these proteins include one subunit of the heterodimeric transferrin receptor (Schell *et al.*, 1991), an alanine-rich protein of unknown function (Nolan *et al.*, 2000), and the variant surface glycoprotein (VSG) (Ferguson *et al.*, 1988) essential for evasion of the host's immune system.

The bloodstream forms of *T. brucei* differentiate into procyclic forms once ingested by the tsetse fly vector. This differentiation involves remodeling of the surface by shed-

ding the VSG coat and replacing it with an invariant coat of GPI-anchored proteins known as procyclins (Roditi *et al.*, 1989). There are four types of procyclin, three bearing between 18 and 30 internal -Glu-Pro- repeats (EP1, EP2, and EP3; see Acosta-Serrano *et al.*, 2000 for alignment), and one with a -Gly-Pro-Glu-Glu-Thr- (GPEET) repeat region (Mowatt *et al.*, 1989). Although EP1 and EP3 both undergo N-glycosylation, EP2 and GPEET do not, although the latter is phosphorylated on the threonine residues of the repeat region (Butikofer *et al.*, 1999). All procyclin isoforms are anchored by a GPI modified with a heterogeneous poly-N-acetyllactosamine side chain (Treumann *et al.*, 1997), widely believed to form a glycocalyx over the cell surface. Displayed above this, both EP and GPEET procyclins are thought to adopt a rod-like conformation (Roditi *et al.*, 1989; Treumann *et al.*, 1997). Although the N-termini of these proteins are proteolytically cleaved during infection of tsetse, their repeat domains are resistant to protease digestion, suggesting that one of the functions of this coat is to act as a mechanical barrier within the proteolytic environment of the tsetse midgut (Acosta-Serrano *et al.*, 2001). A hierarchy

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of procyclin expression during establishment within the tsetse has recently been discovered (Acosta-Serrano *et al.*, 2001). GPEET is the predominant species at day 3 after the blood meal, but there is a switch to glycosylated EP isoforms by day 7. It is known that there is a dramatic decline in parasite numbers during this initial 3 d, whereupon the parasites remaining rapidly multiply to become an established infection with a maximum density of $2\text{--}5 \times 10^5$ parasites/mid-gut (Van den Abbeele *et al.*, 1999).

GPI anchors are synthesized in the endoplasmic reticulum (ER) by the sequential addition of sugars and ethanolamine phosphates onto phosphatidylinositol (for a recent reviews see Ferguson, 1999; Kinoshita and Inoue, 2000). Precursor proteins destined to receive a GPI anchor possess an N-terminal, ER-directing signal sequence that is cleaved upon translocation into the ER and a C-terminal domain comprising a GPI anchor addition signal sequence (Moran and Caras, 1991; Gerber *et al.*, 1992; Nuoffer *et al.*, 1993). This C-terminal domain is recognized by a transamidase complex consisting of at least four subunits (Fraering *et al.*, 2001; Ohishi *et al.*, 2001). The complex proteolytically cleaves the protein at the ω -site and in a transamidation reaction adds a preformed GPI *en bloc* via an amide linkage to the terminal ethanolamine phosphate (Mayor *et al.*, 1991; Maxwell *et al.*, 1995).

Utilization of GPIs for anchoring proteins to the plasma membrane is widespread throughout eukaryotes. GPI anchoring is essential in yeast (Fraering *et al.*, 2001), mammalian embryos (Lin *et al.*, 2000) and bloodstream form *T. brucei* (Nagamune *et al.*, 2000). However, it is not essential in some mammalian cell lines (Yu *et al.*, 1997; Watanabe *et al.*, 2000), in procyclic *T. brucei* (Nagamune *et al.*, 2000) or in the related kinetoplastid parasite *Leishmania mexicana* (Hilley *et al.*, 2000). Although both parasites and their hosts use GPI anchoring, disparity in the substrate specificities of several orthologous GPI biosynthetic enzymes exist (for review, see Ferguson *et al.*, 1999). This raises the possibility that specific inhibitors of parasite GPI biosynthetic enzymes could be designed for use as therapeutic agents in the treatment of disease.

MATERIALS AND METHODS

Identification of *T. brucei* GPI8

A 1.1-kb fragment containing the open reading frame (ORF) of the *L. mexicana* GPI8 (Hilley *et al.*, 2000) was used to screen a *T. brucei* EATRO 795 genomic library under low stringency conditions. A single positive plaque was isolated and DNA was prepared. After restriction endonuclease digestion, an *EcoRI* fragment of ~8.5 kb was subcloned into pUC18 to give pGL480. Sequencing identified a 960-base pair ORF encoding *TbGPI8*.

Southern Blot Analysis

Five micrograms of *T. brucei* EATRO 795 genomic DNA was digested with restriction endonucleases *SacI*, *BamHI*, *EcoRI*, *ApaI*, or *KpnI*, electrophoresed through a 0.7% agarose gel, and blotted onto Hybond C Super (Amersham Pharmacia, Little Chalfant, United Kingdom). The blot was hybridized with a 653-base pair ^{32}P random-primed PCR product (equating to bases 134–787 of the 960-base pair *TbGPI8* ORF, Accession number AJ439686) at 65°C overnight. Washes were for 15 min at 65°C with $2\times$ SSC/0.1% SDS and then twice with $0.2\times$ SSC/0.1% SDS.

Culturing and Transfection of Parasites

Procyclic form *T. brucei* strain EATRO 795 cells were cultured at 27°C in complete SDM79 medium (Brun and Schonenberger, 1979) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum. Five micrograms of linearized DNA was used to transfect 3×10^7 midlog phase procyclics in 0.5 ml Zimmerman PostFusion medium (132 mM NaCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 0.5 mM Mg acetate, 0.09 mM Ca acetate, pH 7.0) in a 0.4-cm pulse cuvette using a Bio-Rad gene pulser II set at 1.5 kV, 25 μF . After overnight recovery, selection of clones was by limiting dilution in nonadherent tissue culture plates (Falcon) with appropriate antibiotics (15 $\mu\text{g ml}^{-1}$ G418, 10 $\mu\text{g ml}^{-1}$ puromycin, 20 $\mu\text{g ml}^{-1}$ blasticidin). Subsequent culturing was in conventional tissue culture flasks.

Bloodstream form *T. brucei* strain 427 cells (SMB; Wirtz *et al.*, 1999) were used for RNAi analysis. These trypanosomes express T7 polymerase and the tet repressor protein that facilitate inducible expression of double-stranded RNA. Cells were cultured at 37°C with 5% CO_2 in HMI-9 (Hirumi and Hirumi, 1989) supplemented with 5 $\mu\text{g ml}^{-1}$ hygromycin and 2.5 $\mu\text{g ml}^{-1}$ G418. Cells (10^7) resuspended in 400 μl Cytomix (25 mM HEPES, 10 mM K_2HPO_4 , 120 mM KCl, 0.15 mM CaCl_2 , 5 mM MgCl_2 , 2 mM EGTA, 1 mM hypoxanthine, 0.5% (wt/vol) glucose, 100 $\mu\text{g ml}^{-1}$ BSA, pH 7.4) were electroporated with 25 μg *NotI* linearized pGL654 using a Bio-Rad Gene Pulser II set at 1.5 kV and 25 μF . Clonal populations were derived by limiting dilution with 2.5 $\mu\text{g ml}^{-1}$ phleomycin. Induction of RNAi expression was with 1 $\mu\text{g ml}^{-1}$ tetracycline.

Plasmids

For deletion of *TbGPI8* the 5' and 3' flanks of the gene were amplified by PCR using primer pairs OL674 (CAAGCTTTTCGCCATCACTCTCAGCCG) with OL675 (AACTAGTCGCCTGATCCAACTAATCG) and OL676 (AGGATCCTTACGATTGTCTAGTTC) with OL677 (AAGATCTCAGCTGTAGACAACCTCAGCG), respectively, and cloned sequentially into the *HindIII/SpeI* and *BamHI/BglIII* sites flanking the PUR^R gene of construct pGL236 (Hilley *et al.*, 2000), giving pGL519. The BSD^R gene was excised from pGL437 (Brooks *et al.*, 2000) using *SpeI/BamHI* and cloned into the same sites of pGL519, replacing the PUR^R gene and giving pGL610. pGL519 and pGL610 were digested with *HindIII/BglIII* and the $\text{PUR}^R/\text{BSD}^R$ -containing fragment purified for transfections. Gene deletion was by homologous recombination, replacing the *TbGPI8* ORF and utilizing the *TbGPI8* 5' and 3' flanking sequences for correct transcription and processing of drug resistance genes.

For reexpression of *TbGPI8*, the ORF together with 449 and 579 base pairs of 5' and 3' flanking sequence, respectively, was amplified by PCR using primer pair OL840 (CAAAGCTTGATCCTTAGATACATACCCG) with OL841 (TGGGATCCACCAGTAACAA-CAGGCAGC) and cloned into the *HindIII/BamHI* sites of plasmid pXS219 (Bangs *et al.*, 1997), giving pGL620. For transfection, pGL620 was linearized with *MluI*, allowing integration into the tubulin intergenic region.

For RNAi, the ORF of *TbGPI8* was amplified by PCR using primer pair OL765b (ACAAGCCTATGTTGCCCATGTTACTGTGG) with OL766 (CAGGATCCCTAGAACAAATCGAACGTAAGTTC) and cloned into the *BamHI* and *HindIII* sites of p2T7i (LaCount *et al.*, 2000), giving pGL654.

Antisera and Immunoblotting

Antiserum specific to GPI8 was raised by immunization of rabbits with purified recombinant *L. mexicana* GPI8 (Sharma *et al.*, 2000). TBRP1/247 monoclonal antiserum against EP-procyclicin was from Cedar lane Laboratories (Ontario, Canada). K1 antiserum against the GPEET-procyclicin (Butikofer *et al.*, 2001) was a kind gift from I. Roditi. Antiserum against the *T. brucei* aldolase was a kind gift from D. Steverding. Antisera against the *T. brucei* variant surface glycoprotein (VSG) 221 were kind gifts from M. Boshart and M. Carrington.

Trypanosome cell pellets were resuspended in Laemmli buffer, electrophoresed on 12% SDS-PAGE gels, and transferred onto PVDF membrane (NEN). Blocking was with 5% (wt/vol) skimmed milk in PBS with 0.1% Tween 20. Primary antibodies for GPI8, EP-procyclicin, and GPEET-procyclicin were used at dilutions of 1:2000, for VSG at 1:5000, and for aldolase at 1:10,000. Secondary antibody (Promega) was conjugated to HRP and was used at 1:2000. Chemiluminescent detection was with the SuperSignal system (Pierce), whereas chemifluorescent detection was with the ECF Western blotting kit and a Typhoon 8600 phosphoimager (Amersham Pharmacia).

Tsetse Fly Infection Study

Procyclic trypanosomes were harvested by centrifugation at $1000 \times g$ for 5 min at 27°C and then resuspended at 10^6 ml^{-1} in red blood cells washed with heat-inactivated fetal calf serum. Tsetse flies were fed through a silicon membrane on a 37°C plate and then maintained at 25°C with 65% humidity for 14 d with feeding every 2 d. After this time, they were dissected and their midguts examined for the presence of trypanosomes. Images were captured using a Zeiss Axioplan at $\times 630$ magnification with a Hamamatsu C4742–95 cooled digital CCD camera and processed using Openlab 2.02.

Metabolic Labeling and Extraction of Parasite Material

For metabolic labeling, 10^8 procyclic parasites were harvested at $1000 \times g$ for 5 min at 27°C and washed twice with PBS. The parasites were resuspended in 10 ml SDM79 containing 250 μCi [$1\text{-}^3\text{H}$]ethanolamine hydrochloride (Amersham) for 16 h or in 5 ml glucose-free RPMI containing 200 μCi D-[$2,6\text{-}^3\text{H}$]mannose (Amersham) for 5 h. For periodate treatment, D-[$2,6\text{-}^3\text{H}$]mannose-labeled procyclics were resuspended in 20 ml SDM79 for 4 h, washed twice with ice-cold PBS and then resuspended in 10 ml ice-cold PBS. Samples were split and NaIO_4 added to one of the matched pair to 10 mM final concentration, after which samples were incubated on ice for 30 min in the dark. Cells were washed twice with ice-cold PBS/150 mM glycerol and twice with ice-cold PBS, and pellets were stored at -80°C .

Lipid extracts were prepared as described previously (Field *et al.*, 1991b). After partitioning to remove aqueous-soluble metabolites, organic phases were dried down in a Speedvac (Savant) and resuspended in 20 μl solvent. Ten microliters of each sample was spotted on a Si60 HPTLC plate (Merck) and then developed in chloroform/methanol/water (10:10:3, vol/vol) in a saturating atmosphere. The total migration distance was 16 cm. After development, the plates were air dried, sprayed with EnHance (Perkin Elmer-Cetus), and exposed to autoradiographic film at -85°C . To generate a PP1* standard, PP1 was purified by elution from Si60 TLC plates (Merck) after chromatography in chloroform/methanol/water (10:10:3, vol/vol). After several rounds of extraction in chloroform/methanol/water (10:10:3, vol/vol), samples were dried and repartitioned between butanol/water, and then lipids located by scintillation counting of an aliquot. Periodate treatment was done in 250 μl 40% propan-1-ol, 20% MeOH, 40% PBS, with 10 mM periodate on ice in the dark for 10 min. Reactions were stopped by mixing with an equal volume of 150 mM glycerol in PBS, and samples were chromatographed as described above.

RESULTS

Isolation of *T. brucei* GPI8

Use of the ORF of the *L. mexicana* GPI8 to screen a *T. brucei* λ library resulted in a positive plaque being isolated and 1.6 kb of sequence being obtained. This identified a 960-base pair ORF (*TbGPI8*) encoding the *T. brucei* GPI8. *TbGPI8* is predicted to be a 37-kDa protein, with a putative signal peptide cleavage site between positions 20 and 21. Compar-

ison of *TbGPI8* with the protein from other organisms (Figure 1) reveals 49% identity with *L. mexicana* and 27% identity with both *Saccharomyces cerevisiae* and *Homo sapiens*. *TbGPI8* contains the catalytic histidine and cysteine dyad (marked in Figure 1) that defines the clan CD cysteine proteases (Barrett and Rawlings, 2001). Hydrophathy analysis revealed *TbGPI8* lacks the C-terminal transmembrane domain reported for *S. cerevisiae* and *H. sapiens* (Benghezal *et al.*, 1996). Amplification of *TbGPI8* from cDNA using a gene internal and a spliced leader-specific primer pair identified the splice acceptor site 304 base pairs 5' of the start codon.

Procyclic Form Δgpi8 Trypanosomes Lack GPI-anchored Proteins

Southern blot analysis of endonuclease-digested genomic DNA (Figure 2A) revealed a single *GPI8*-hybridizing DNA fragment with *KpnI*, *ApaI*, *EcoRI*, or *BamHI*. Two DNA fragments were detected with *SacI* because of the presence of a *SacI* site near the middle of the gene. These data show that the *T. brucei* *GPI8* is a single-copy gene. To investigate the importance of GPI8 in procyclic *T. brucei*, sequential rounds of targeted gene replacement with *PAC* and *BSD* antibiotic resistance genes flanked by *GPI8* 5' and 3' sequences were performed (Figure 2B). After PCR confirmation of *PAC* integration at the correct locus, the second allele of *GPI8* was deleted from two independent clones using the *BSD*-containing construct. Clonal populations were derived, and deletion of *GPI8* (to give Δgpi8) was confirmed by both PCR (Figure 2C) and Southern blot (unpublished data). The *T. brucei* *GPI8* was targeted into the tubulin locus of the Δgpi8 mutants to generate lines reexpressing GPI8 (designated $\Delta\text{gpi8}[\text{GPI8}]$). Procyclic form wild-type, Δgpi8 and $\Delta\text{gpi8}[\text{GPI8}]$ were found to have no discernible difference in either morphology or growth rate in culture.

Western blot analysis of whole cell lysates revealed abundant EP and GPEET procyclins in both wild-type and $\Delta\text{gpi8}[\text{GPI8}]$ clones, but their apparent absence from Δgpi8 (Figure 3A). This suggested that GPI-anchored proteins were not present in trypanosomes that lack *GPI8*. However, a recent report has indicated that both of the antibodies against procyclins used in the current study (TBRP1/247 and K1) fail to detect procyclins that no longer possess GPI anchors (Butikofer *et al.*, 2001). Thus it was possible that non-GPI anchored procyclins could still associate with the surface of Δgpi8 cells by means of the hydrophobic C-terminal domain normally cleaved during the GPI:protein transamidation reaction. To investigate this possibility, a previously reported method (Clayton and Mowatt, 1989) was used to hypotonically lyse cells in the presence of protease inhibitors and then extract protein from the membrane fraction using CHAPS. After PAGE, Stainsall was used to detect protein within the gel. Procyclicin stained a deep blue in both wild-type and $\Delta\text{gpi8}[\text{GPI8}]$ clones, but was not observed in Δgpi8 (Figure 3B). This confirmed that procyclicin was not expressed on the cell surface in the absence of GPI anchoring. Sypro Ruby staining of the CHAPS extracts showed that there were a number of proteins expressed in both wild-type and $\Delta\text{gpi8}[\text{GPI8}]$ trypanosomes that were apparently absent from Δgpi8 (Figure 3B). Also, a subset of proteins appeared to be expressed at much higher levels in Δgpi8 cells relative to both wild-type and $\Delta\text{gpi8}[\text{GPI8}]$.

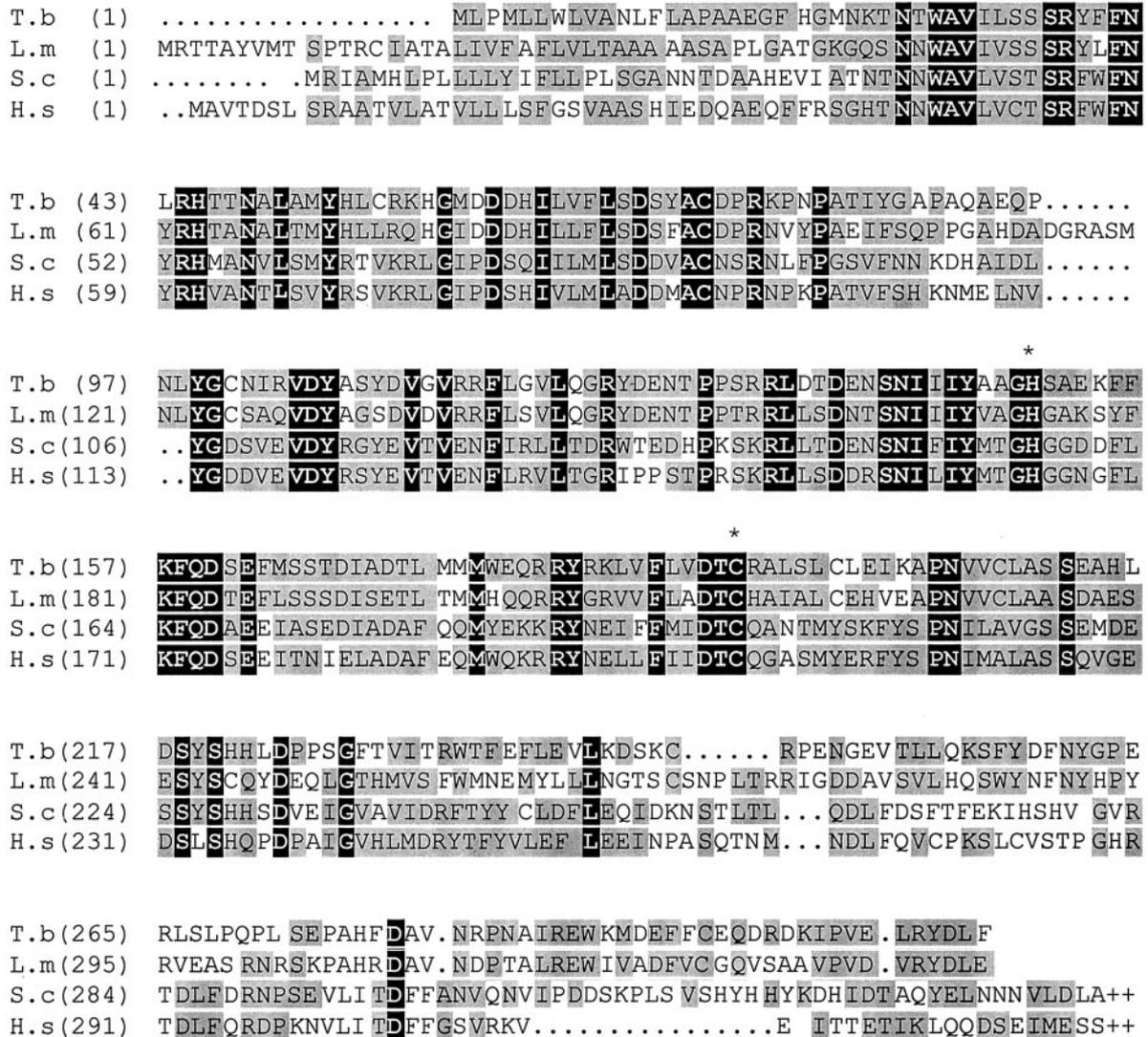


Figure 1. Multiple alignment of GPI8 amino acid sequences from *T. brucei* (this study), *Leishmania mexicana* (CAB55340), *S. cerevisiae* (NP_010618) and *Homo sapiens* (CAA68871). The alignment was performed using Align X (Vector NTI Suite, Informax Inc.). Amino acids identical in all sequences are highlighted with a black background, and gray blocks denote conservation. Active site cysteine and histidine residues are indicated by an asterisk (*).

Effect of ConA on Procyclic Δ *gpi8*

The tetrameric lectin concanavalin A has a strong affinity for mannose residues and binds to the N-linked glycans of EP-procyclics, resulting in procyclic cell death. Cells bearing procyclic isoforms that are either not glycosylated or have altered N-glycans are resistant to ConA-induced death (Pearson *et al.*, 2000). We therefore reasoned that because Δ *gpi8* cells lack procyclicin on the cell surface, then they would be resistant to ConA-induced cell death. To test this hypothesis, wild-type, Δ *gpi8*, or Δ *gpi8*[GPI8] cells were cultured in medium containing 50 μ g ml⁻¹ ConA. All three clones showed the same kinetics of cell death, including the characteristic morphology previously described (Welburn *et al.*, 1996; Pearson *et al.*, 2000). This indicates the presence of other mannose-bearing structures that replace procyclicin on

the surface of procyclic Δ *gpi8* as ligands to which ConA can bind.

Procyclic Δ *gpi8* Accumulate GPI Anchor Precursors

Analysis of chloroform/methanol/water (CMW) extracts from both ³H-ethanolamine- and ³H-mannose-labeled procyclic forms by TLC revealed that although wild-type EATRO 795 and Δ *gpi8*[GPI8] display lipid profiles that are essentially identical, there is an increase in the abundance of ethanolamine- and mannose-containing lipids in Δ *gpi8* cells (Figure 4A). Designation of lipid species is made based on R_f values from previous experiments (Field *et al.*, 1991b). PP1, the complete preformed GPI substrate of the transamidase complex of procyclic *T. brucei* (Field *et al.*, 1991a; Mayor *et al.*,

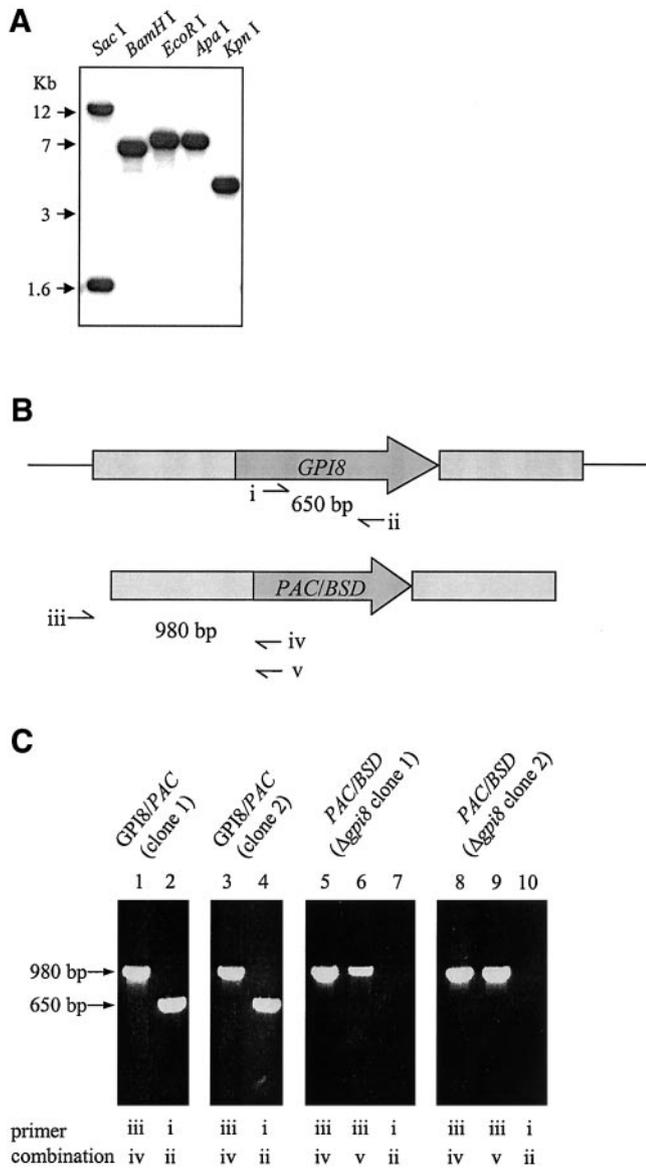


Figure 2. Generation of $\Delta gpi8$ mutants (A) Southern blot analysis of the *T. brucei* *GPI8* gene. DNA was cut with *SacI*, *BamHI*, *EcoRI*, *ApaI*, or *KpnI*, transferred onto nylon membrane and hybridized with the ORF of the gene. (B) Schematic representation of the *GPI8* locus (top) and *PAC/BSD* replacement construct (bottom) with open reading frames (dark gray) and *GPI8* flanking regions (light gray) used in the integration construct. Thin lines represent regions not present in the integration construct. Primer sites used to confirm presence of *GPI8* (i and ii) and correct integration of *PAC/BSD* constructs (iii with iv or v) are indicated. (C) PCR of genomic DNA to determine correct integration of markers and deletion of *GPI8*. *GPI8/PAC* (clone 1) and (clone 2) are two independent heterozygotes containing a *PAC* (lanes 1 and 3) and a *GPI8* (lanes 2 and 4) gene. Derived from these are two independent $\Delta gpi8$ clones, containing *PAC* (lanes 5 and 8) and *BSD* (lanes 6 and 9), but lacking *GPI8* (lanes 7 and 10). $\Delta gpi8$ clone 1 was used for all subsequent studies. Some results were confirmed with clone 2 (unpublished data).

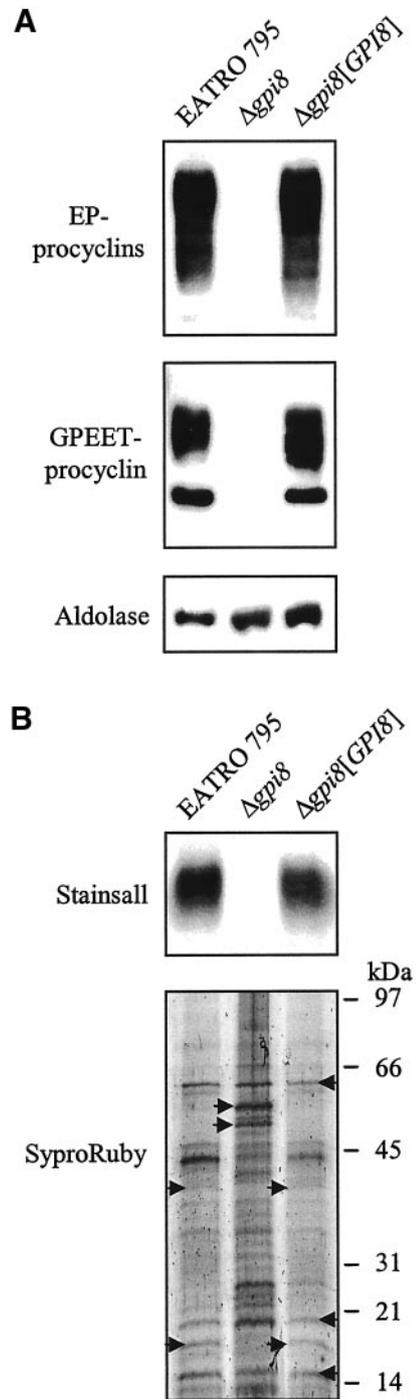


Figure 3. Analysis of procyclin expression (A) Western blot analysis of whole cell lysate from wild-type EATRO 795, $\Delta gpi8$ and $\Delta gpi8[GPI8]$ detected with α -EP procyclin, α -GPEET procyclin, or α -aldolase. (B) Wild-type EATRO 795, $\Delta gpi8$, and $\Delta gpi8[GPI8]$ were hypotonically lysed, and the washed pellet was subjected to CHAPS extraction. After SDS-PAGE, CHAPS extracted protein was detected with Stainsall or SyproRuby. Arrows indicate abundant proteins present in all three samples (\leftarrow) or predominantly in the wild-type EATRO 795 and $\Delta gpi8[GPI8]$ or $\Delta gpi8$ (\rightarrow).

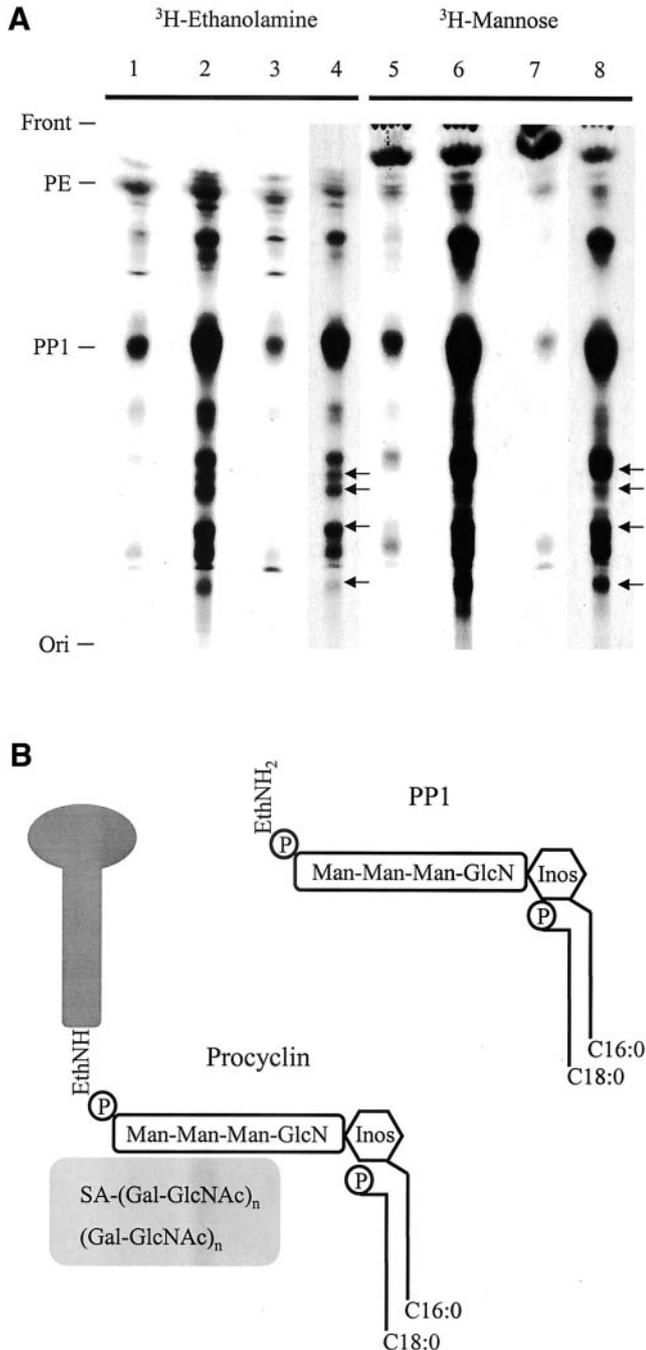


Figure 4. Accumulation of lipids in $\Delta gpi8$. (A) Chloroform/methanol/water extracts of ^3H -ethanolamine- and ^3H -mannose-labeled procyclic forms were chromatographed and visualized by autoradiography. $\Delta gpi8[GPI8]$ (lanes 1 and 5), $\Delta gpi8$ (lanes 2, 4, 6, and 8), and wild-type EATRO 795 (lanes 3 and 7). The positions of the front, origin, phosphatidylethanolamine (PE), and the major insect-stage *T. brucei* GPI-anchor precursor PP1 (Field *et al.*, 1991b) are indicated at left. A number of novel lipid species were detected in $\Delta gpi8$ (indicated by arrows [←]). For clarity, the $\Delta gpi8$ extracts are shown after both 2-d (lanes 4 and 8) and 10-d (lanes 2 and 6) exposures. (B) Schematic illustrating the structural features of PP1 and the procyclin GPI anchor. Data are based on studies cited in the text. PP1, top

1991), was particularly abundant in cells lacking GPI:transamidase activity. The structure of PP1 and the procyclin GPI anchor are shown (Figure 4B). The accumulation of GPI-precursors was due to the loss of GPI:protein transamidase activity, as reexpression of GPI8 in $\Delta gpi8[GPI8]$ restored the lipid profile to that of the wild-type parasites. In addition to the build up of previously observed precursors, a number of novel ethanolamine/mannose-containing lipid species also accumulated in $\Delta gpi8$ (Figure 4A [←]). This indicates that in the absence of addition to proteins a significant proportion of the anchor pool has undergone further modification. It is feasible that these novel lipid species are present at low levels in wild-type cells, but are difficult to detect because of the relatively low PP1 pool.

Many eukaryotes express non-protein-linked GPI molecules on their plasma membranes. These include the lipophosphoglycans and GIPLs of *Leishmania* and 'free' GPI anchors in mammalian cells (McConville and Ferguson, 1993; Baumann *et al.*, 2000). We therefore speculated that *T. brucei* may express free GPI molecules on the extracellular face of the plasma membrane and that the marked accumulation of GPI molecules detected in $\Delta gpi8$ cells could be indicative of an amplification of this phenomenon. To determine if this were the case, we utilized a previously published method for identifying expression of free GPIs on the exoplasmic leaflet of the plasma membrane (Baumann *et al.*, 2000). Sodium periodate oxidizes the hydroxyl groups of sugars, but because of its size and negative charge does not diffuse into cells (McConville and Bacic, 1990; Baumann *et al.*, 2000). $\Delta gpi8$ were labeled with ^3H -mannose and after a chase period incubated in the presence of 10 mM NaIO_4 . CMW extracts analyzed by TLC revealed that after periodate treatment a significant portion of the PP1 fraction had been oxidized (PP1*), indicating that part of this pool is expressed on the surface of these parasites (Figure 5A, lanes 1 and 2). As a control, PP1 was purified by elution from a TLC plate and treated with periodate to provide an authentic PP1* standard (lanes 3 and 4). To show that periodate did not permeate the plasma membrane and oxidize intracellular PP1, $\Delta gpi8$ cells were labeled for 2.5 h with ^3H -mannose and then treated with periodate as described above. No substantial change in lipid profile was detected between the treated and untreated samples (compare Figure 5B, lanes 1 and 2). Once extracted from $\Delta gpi8$ cells, all PP1 converted to PP1* upon incubation with periodate (lanes 3 and 4). Additionally, a glycerol quench applied before periodate treatment ensured that no oxidation occurred during the exper-

Figure 4 (cont). right, contains a canonical GPI-core glycan, together with an acyl-inositol headgroup (the major fatty acid here is palmitate, although other species are present) and a *lyso*-glycerolipid backbone (a single fatty acid, predominantly stearate, is attached to the glycerol *sn*-1 position). The procyclin GPI-anchor structure has the same core as PP1, including an identical fatty acid configuration. Additionally, the procyclin protein C terminus is in amide linkage to the ethanolamine, and there is a large heterogeneous glycan of ~15-kDa molecular weight, which contains sialic acid and is endo- β -galactosidase sensitive (indicating the presence of lactosamine repeat structures). Inositol is represented by a hexagon, the core glycan, and the side chain in the procyclin anchor by rounded rectangles, and the procyclin polypeptide by a lollipop. Diagram is not to scale.

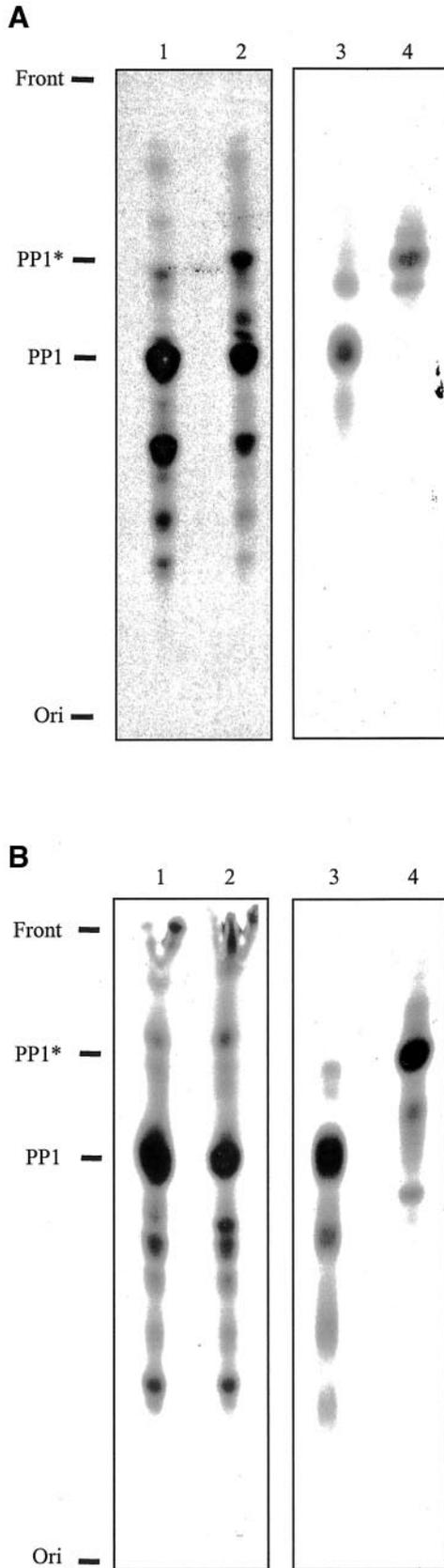


Table 1. Wild-type EATRO 795, $\Delta gpi8$ and $\Delta gpi8[GPI8]$ were fed to teneral tsetse flies as part of a blood meal

Clone	EATRO 795	$\Delta gpi8$	$\Delta gpi8[GPI8]$
Expt 1	*	1/39	11/33
Expt 2	24/49	1/66	9/20
Expt 3	29/59	*	24/65
Collated data	53/108	2/105	44/118
% infected	49	2	37

Each study was carried out at least twice as indicated, dependent on availability of tsetse. Mid-gut dissections were carried out after 14 d. For each experiment, the number of flies harboring parasites/total number of flies dissected is shown. Collated % infection is shown at the bottom.

imental procedure (unpublished data). These data provide evidence that a fraction of the PP1 pool of $\Delta gpi8$ cells is surface located.

***Δgpi8* Do Not Establish a Successful Infection in Tsetse**

The repeat domains of *T. brucei* procyclins are known to be resistant to digestion by tsetse proteases (Acosta-Serrano *et al.*, 2001), whereas their GPI anchors are modified with a large poly-*N*-acetylactosamine side chain (Treumann *et al.*, 1997). This has led to the hypothesis that one of the major functions of the procyclin coat is to form a glycocalyx, which provides the cell with a protective barrier against the digestive enzymes of the tsetse fly midgut (Ferguson *et al.*, 1993; Ruepp *et al.*, 1997). In the current study, wild-type, $\Delta gpi8$ or $\Delta gpi8[GPI8]$ cells were fed to teneral tsetse flies as part of a blood meal, with midgut dissection of flies 14 d later. Wild-type and $\Delta gpi8[GPI8]$ procyclics infected 49 and 37% of flies, respectively, whereas $\Delta gpi8$ infected <2% (Table 1). Furthermore, both of the flies infected by $\Delta gpi8$ harbored very sparse populations of parasites, and these cells resembled cultured procyclic forms rather than the more serpentine procyclic forms that characterize established midgut infections (Figure 6). Because $\Delta gpi8[GPI8]$ cells behaved in a

Figure 5. Surface expression of protein-anchor precursors. (A) $\Delta gpi8$ cells labeled with 3H -mannose were chased for 4 h in fresh medium then incubated without (lane 1) or with (lane 2) 10 mM sodium periodate. After quenching of the oxidation reaction, cell pellets were extracted sequentially with chloroform/methanol and chloroform/methanol/water, chromatographed, and visualized by autoradiography. The chloroform/methanol fraction demonstrated even loading of samples as determined by phosphatidylethanolamine abundance. The positions of the front, origin, PP1, and oxidized PP1 (PP1*) in the chloroform/methanol/water fraction are indicated on the left. To provide an authentic standard for PP1*, PP1 was purified by elution from a TLC plate and treated without (lane 3) and with (lane 4) 10 mM periodate for 10 min. Samples were chromatographed and visualized by autoradiography. (B) $\Delta gpi8$ cells labeled for 2.5 h with 3H -mannose and then incubated without (lane 1) or with (lane 2) 10 mM sodium periodate. Lipids extracted from $\Delta gpi8$ cells were incubated in the absence (lane 3) or presence (lane 4) of 10 mM periodate for 10 min. Samples were chromatographed as in A.

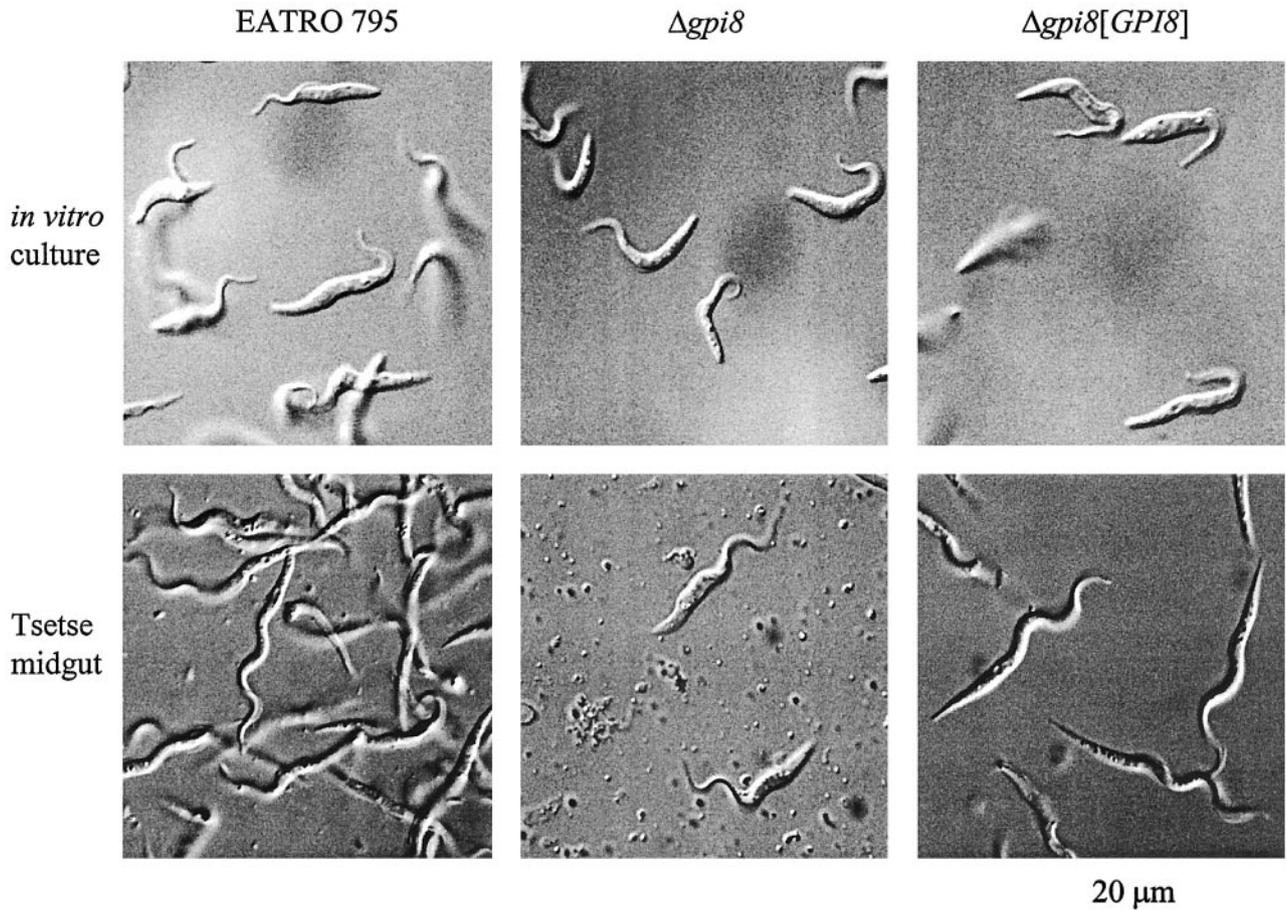


Figure 6. Tsetse fly infectivity. Wild-type EATRO 795, $\Delta gpi8$, and $\Delta gpi8[GPI8]$ were fed to teneral tsetse flies as part of a blood meal. After 14 d, midguts were dissected and examined microscopically. Images of living parasites from in vitro cultures and tsetse dissections are displayed.

manner similar to the wild-type parasites, both in rate of infection and midgut morphology, failure of $\Delta gpi8$ trypanosomes to establish in tsetse must be due to the lack of GPI:protein transamidase activity.

GPI8 Is Essential for Cell Cycle Progression in Bloodstream form Trypanosomes

We used an inducible RNAi approach (Ngo *et al.*, 1998; Wang *et al.*, 2000) to investigate the requirement for GPI8 in bloodstream trypanosomes. Culturing cells in the presence of tetracycline-induced expression of double-stranded *GPI8* RNA. A severe growth deficit was observed in induced cells when compared with uninduced controls (Figure 7A). Northern blotting confirmed that *GPI8* mRNA levels were reduced significantly in induced cells relative to a *MOB1* control (Figure 7B), whereas Western blotting demonstrated GPI8 to be apparently absent (Figure 7C). Interestingly, *GPI8* RNAi induction resulted in a 10% increase in the abundance of VSG (Figure 7C) in comparison to an adolase control, leading us to speculate that accumulation of unanchored precursor proteins may be occurring as a

result of reduced GPI anchoring. To address this question, cells cultured for 24 h $-/+$ induction were hypotonically lysed and then incubated at 37°C for 30 min. This treatment allowed the endogenous trypanosomal GPI-PLC to hydrolyze the GPI anchor of VSG, converting the glycoprotein from a membrane bound to a water-soluble form (De Almeida and Turner, 1983). As a control, cells were lysed in the presence of 10 mM ZnCl_2 , a known inhibitor of GPI-PLC. After ultracentrifugation, P100 and S100 fractions were analyzed by Western blotting with α -VSG 221 or with α -aldolase as a loading control. In uninduced cells, GPI-PLC activity resulted in all VSG being found in the S100 fraction as had been predicted. By contrast, RNAi induction resulted in the accumulation of proVSG, which was resistant to GPI-PLC activity and hence remained associated with membranes.

Microscopic analysis revealed that by 12 h post-induction cells had begun to swell and lose motility and that after 18 h the morphology of many was radically altered. DAPI staining revealed that the majority of these cells had karyotype defects (Monsters), with multiple nuclei, kinetoplasts, and

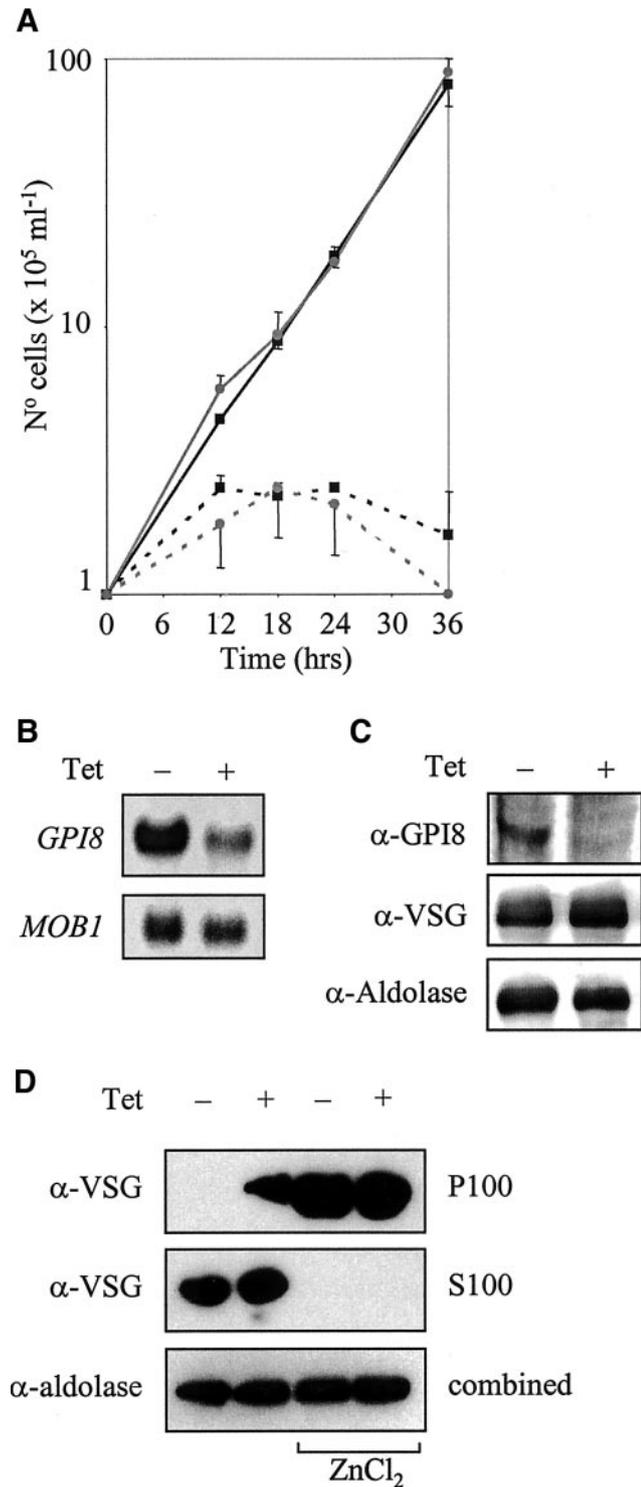


Figure 7. RNAi of bloodstream form trypanosomes. (A) Growth curve of two independently derived clones in the absence (solid line) or presence (dashed line) of $1 \mu\text{g ml}^{-1}$ tetracycline. The experiment was carried out in triplicate and bars denoting SD are shown. (B) Northern blots of total RNA prepared from parasites 12 h $-/+$ induction, electrophoresed on a 1% agarose gel and transferred onto

flagella (Figure 8). This suggests that lack of GPI:protein transamidase activity results in perturbation of cell cycle control, leading to cell death.

DISCUSSION

The *GPI8* gene of *T. brucei* encodes a predicted protein with significant sequence identity to GPI8 from *L. mexicana*, *S. cerevisiae*, and *H. sapiens*. However, although GPI8 is a type I ER membrane protein in both *S. cerevisiae* and *H. sapiens*, hydropathy analysis indicates that the trypanosome GPI8 lacks a C-terminal transmembrane domain. This is consistent both with the hydropathy profile of the GPI8 of the closely related trypanosomatid *L. mexicana* (Hilley *et al.*, 2000; Sharma *et al.*, 2000) and our previous studies that indicated that trypanosomal GPI8 is a soluble ER protein (Sharma *et al.*, 2000). Deletion of *GPI8* from procyclic *T. brucei* resulted in parasites deficient in GPI-anchored proteins (Figure 3) and with an accumulation of precursor GPIs (Figure 4A), confirming that GPI8 is a functional component of the GPI:protein transamidase of *T. brucei*. The parasite does not have an alternative pathway for GPI anchor addition to proteins, making the GPI8 cysteine protease essential for the production of GPI-anchored proteins.

A previous study demonstrated that disruption of *Tb-GPI10*, which encodes the third mannosyltransferase in GPI anchor biosynthesis, resulted in procyclic cells with an *in vitro* doubling time approximately twice that of their wild-type counterpart and a requirement for nonadherent culture conditions (Nagamune *et al.*, 2000). Surprisingly, despite their reduced fitness *in vitro*, these mutants retained their ability to establish infection within the midgut of tsetse, albeit with reduced efficiency. As both GPI8 and GPI10 are essential for GPI anchoring of proteins, we expected that deletion of *GPI8* would result in a phenotype that mimicked *GPI10* null mutants. However, the current study has demonstrated that the *GPI8* and *GPI10* null mutants differ in several key respects. Although both have been shown to lack GPI-anchored proteins, Δgpi8 did not require nonadherent culture conditions for growth, had a cell cycle of comparable length to their wild-type parent, and yet were unable to establish infection within the tsetse fly midgut. Comparison of metabolically labeled GPI lipids from these two mutants provides an explanation for these differences. Δgpi8 accumulated PP1, the complete preformed GPI substrate of the transamidase complex of procyclic *T. brucei* (Mayor *et al.*, 1991; Field *et al.*, 1991a). This is in keeping with similar data from *L. mexicana* (Hilley *et al.*, 2000), *S. cerevisiae* (Benghezal

Figure 7 (cont). nylon membrane. Duplicate blots were hybridized with ^{32}P -labeled ORFs of either *GPI8* or *MOB1*. (C) Western blot analysis of whole cell lysate 24 h $-/+$ induction probed with α -GPI8, α -VSG 221, or α -aldolase. A Typhoon 8600 phosphoimager was used for detection and quantitation. (D) Cells, 1×10^7 , cultured for 24 h $-/+$ induction were lysed on ice in $25 \mu\text{l}$ 0.05% TX-100 with 1 mg ml^{-1} pefabloc and $1 \mu\text{g ml}^{-1}$ leupeptin and then incubated at 37°C for 30 min. Alternatively cells were lysed as above in the presence of 10 mM ZnCl_2 and maintained on ice for 5 min. Lysates were made up to 1 ml, after which they were centrifuged at $100,000 \times g$ for 30 min at 4°C to yield P100 and S100 fractions. Samples were analyzed by Western blotting with α -VSG 221 or α -aldolase.

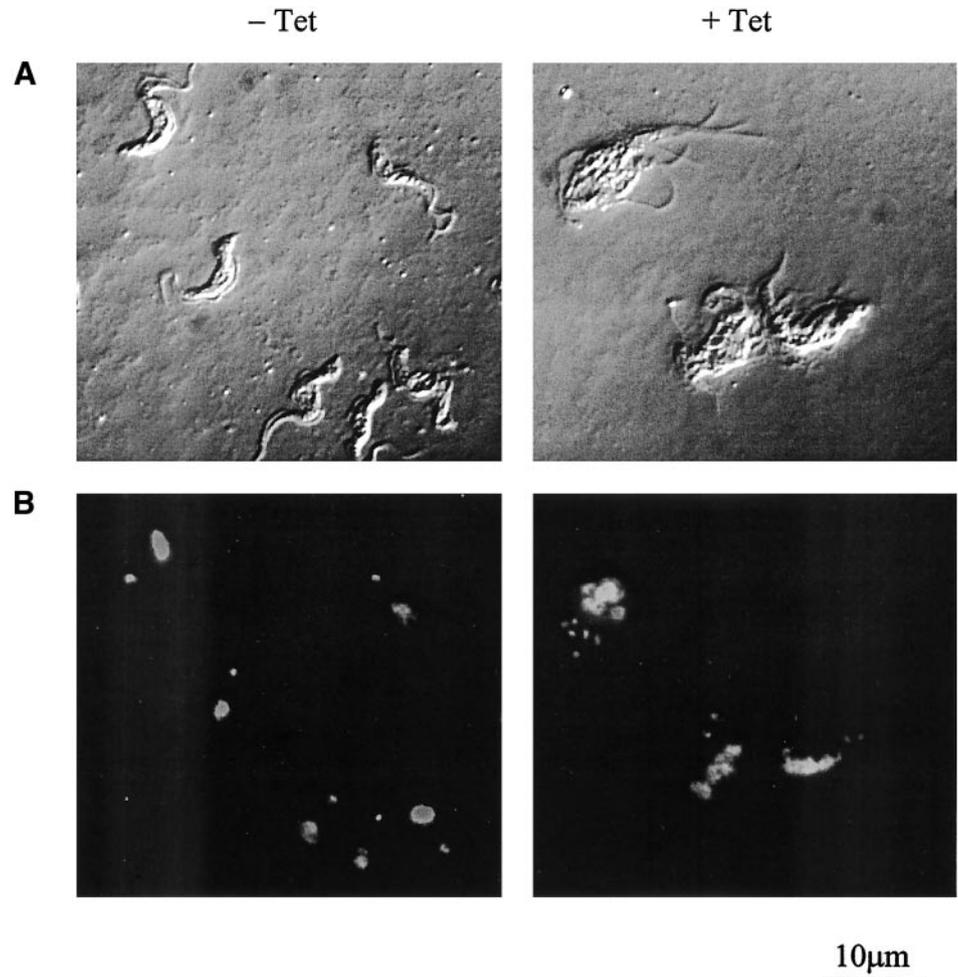


Figure 8. Microscopic analysis of cultured bloodstream form parasites without (-) or with (+) 18 h tetracycline induction of *GPI8* RNAi using (A) phase contrast or (B) DAPI staining of DNA.

et al., 1996), and human (Yu *et al.*, 1997), all of which accumulate mature GPI in the absence of GPI8 function. Furthermore, our data indicate that $\Delta gpi8$ cells express part of the accumulated PP1 pool on their plasma membrane. $\Delta gpi10$, by contrast, did not synthesize complete GPI anchors and accumulated different mannosylipids than wild-type cells (Nagamune *et al.*, 2000). They could not express the free surface GPIs we report here, therefore explaining their reduced fitness *in vitro*.

Expression of free GPIs on the surface of eukaryotic cells is not without precedent (McConville and Ferguson, 1993; Baumann *et al.*, 2000) and has also been discovered recently in "naked" procyclic trypanosomes deficient in procyclin (E. Vassella, personal communication). A number of novel lipid species were found to build up in the $\Delta gpi8$ mutant, which were not detected in the wild-type (Figure 4A). These may be a result of the accumulating lipids being acted upon by the normal biosynthetic pathway that decorates lipid anchors in the Golgi and, if this is the case, these lipids are unlikely to be substrates for the GPI:protein transamidase, which is located in the ER. The possibility of free GPIs on the surface explains our observation that Con A was capable of killing $\Delta gpi8$ cells. Previous studies have shown that Con A binds to the N-linked glycans of EP procyclins 1 and 3,

resulting in a procyclic cell death phenotype that has been likened to apoptosis in metazoan cells (Welburn *et al.*, 1996). Clones that exclusively express the procyclins EP2 and GPEET, both of which lack N-glycosylation sites and are not bound by ConA, are resistant to this form of cell death (Pearson *et al.*, 2000). The plasma membrane of $\Delta gpi8$ cells lack all procyclin, but have the tri-mannose GPI, PP1, which is capable of binding ConA (Vidugiriene and Menon, 1994). Although Con A-induced death of wild-type procyclic parasites is mediated through interaction of the lectin with procyclin-borne glycans, the actual mechanism by which this kills the cells is not understood. It is therefore entirely feasible that interaction of Con A with the free GPIs of $\Delta gpi8$ cells could elicit the same cellular response. Interestingly, we have noted previously that there are two pools of PP1 in wild-type parasites, one pool that is turned over with rapid kinetics ($t_{1/2} = 8$ h) and a more stable pool (M. Field, unpublished). The presence of two pools of PP1 is consistent with the concept that a portion of PP1 is located on the surface of wild-type parasites as well as $\Delta gpi8$.

Surface expression of free GPIs by $\Delta gpi8$ cells also provides an explanation for our observation that these mutants cannot establish an infection in the tsetse midgut. One of the proposed functions of the GPI-anchored procyclin coat of *T.*

brucei is provision of cells with a protease-resistant glycoalyx, enabling them to survive within the lumen of the tsetse fly midgut. Deletion either of EP procyclin genes (Ruepp *et al.*, 1997) or *GPI10* (Nagamune *et al.*, 2000) resulted in reduced ability of procyclic cultures to establish infections within tsetse, supporting this view. However, although reduced infection frequencies were reported in both of these studies, heavy infections were still observed. By contrast, the current study detected Δ *gpi8* cells in only 2% of flies dissected and with very low parasitaemia and abnormal morphology in both cases. This suggests that tsetse flies are essentially refractory to the successful establishment of Δ *gpi8*. Little is known about the molecular basis for refractoriness of tsetse to trypanosome infection. There is some circumstantial evidence to suggest that tsetse flies possess a trypanocidal midgut lectin (Maudlin and Welburn, 1987; Welburn *et al.*, 1994), although this lectin has never been purified and characterized. Such a lectin could be responsible for the inability of Δ *gpi8* parasites to establish infection within the midgut of the vector, with the tri-mannose core of their exposed GPI coat being bound by the lectin. Δ *gpi10* mutants by contrast lack this free GPI on their surface and would not be susceptible to agglutination. An alternative explanation is that the surface architecture of the procyclic form will differ between Δ *gpi8* and Δ *gpi10* mutants because of the variation in their free GPIs. Such differences may alter their susceptibility to other tsetse immune responses, such as the antimicrobial peptides attacin, defensin, and dipteracin (Hao *et al.*, 2001).

The Δ *gpi8* cells that were detected in the midguts of the two tsetse were morphologically indistinguishable from counterparts grown in *in vitro* cultures. By contrast, wild-type and Δ *gpi8*[*GPI8*] parasites observed in the midguts had all assumed the phenotype characteristic of posterior midgut procyclic trypomastigotes (as described in Van den Abbeele *et al.*, 1999). This shows that the failure to differentiate was a direct result of the *GPI8* deletion. However, such a phenotype was not reported with the Δ *gpi10* mutants, suggesting that it is not simply a consequence of the lack of GPI-anchored proteins.

RNAi was used to show that *GPI8* is essential in bloodstream form trypanosomes grown in *in vitro* culture. Northern and Western blot data demonstrated specific down-regulation of both *GPI8* mRNA and protein, with concomitant accumulation of proVSG. Recently it has been shown that proVSG with an altered GPI-anchor signal sequence accumulates within the ER and is then degraded (Bohme and Cross, 2002). Our data provide direct evidence to support more circumstantial data (Nagamune *et al.*, 2000) that expression of GPI-anchored proteins are essential for bloodstream form trypanosomes. Cells with reduced *GPI8* rapidly lost viability, displaying obvious dysfunction in cell cycle progression with an accumulation of cells with multiple nuclei, flagella, and kinetoplasts. This phenotype is consistent with a block in cytokinesis. Studies in our laboratory have indicated that trypanosomes lack certain key cell cycle checkpoints that are present in most eukaryotes. In particular, RNAi of the mitotic cyclin CYC6 in bloodstream form trypanosomes led to a block in mitosis but allowed reinitiation of nuclear and kinetoplast S-phase (Hammarton and Mottram, in preparation). For the *GPI8* RNAi mutants a block in cytokinesis does not appear to prevent kinetoplast

and nuclear replication or subsequent organelle segregation, reinforcing the finding that initiation of S-phase can occur in the absence of completion of cytokinesis. Cell cycle arrest in response to a defect in GPI biosynthesis has been reported previously in *S. pombe* (Colussi and Orlean, 1997). It has been hypothesized that this phenomenon in yeast could be the result of inefficient delivery of GPI-anchored proteins involved in cell division to their site of action. Depletion of GPI anchors in *Trypanosoma cruzi* by heterologous expression of *T. brucei* GPI-PLC lead to an apparent block in mitosis (Garg *et al.*, 1997). In *T. brucei*, it is possible that reduction or ablation of GPI-anchored receptors (e.g., transferrin) or other GPI-anchored protein such as BARP (Nolan *et al.*, 2000) may render cells incapable of undergoing cytokinesis. Alternatively, it is possible that accumulation of GPI precursors, or intracellular proVSG, results in perturbation of the cell cycle or that VSG occupancy of the plasma membrane is required at a given density. In the later scenario, reduction in the rate at which VSG is anchored and trafficked to the cell surface could negatively impact on the rate at which membrane is synthesized, resulting in cells being unable to undergo cytokinesis at a time when mitosis has been initiated. Whatever the cause, the current work clearly demonstrates that reduced GPI:protein transamidase activity results in a cytokinesis block in bloodstream form trypanosomes.

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