Original Article

Identification of a glycosylphosphatidylinositol anchor-modifying β1-3 galactosyltransferase in *Trypanosoma brucei*

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Abstract

*Trypanosoma brucei* is the causative agent of human African sleeping sickness and the cattle disease nagana. *Trypanosoma brucei* is dependent on glycoproteins for its survival and infectivity throughout its life cycle. Here we report the functional characterization of *TbGT3*, a glycosyltransferase expressed in the bloodstream and procyclic form of the parasite. Bloodstream and procyclic form *TbGT3* conditional null mutants were created and both exhibited normal growth under permissive and nonpermissive conditions. Under nonpermissive conditions, the normal glycosylation of the major glycoprotein of bloodstream form *T. brucei*, the variant surface glycoprotein and the absence of major alterations in lectin binding to other glycoproteins suggested that the major function of *TbGT3* occurs in the procyclic form of the parasite. Consistent with this, the major surface glycoprotein of the procyclic form, procyclin, exhibited a marked reduction in molecular weight due to changes in glycosylphosphatidylinositol (GPI) anchor side chains. Structural analysis of the mutant procyclin GPI anchors indicated that *TbGT3* encodes a UDP-Gal: β1→4GlcNAc-GPI β1→3 Gal transferase. Despite the alterations in GPI anchor side chains, *TbGT3* conditional null mutants remained infectious to tsetse flies under nonpermissive conditions.

Key words: galactosyltransferase, GPI-anchor, procyclic form, *TbGT3*, *Trypanosoma brucei*

Introduction

The tsetse-transmitted *Trypanosoma brucei* group of parasites cause Human African Trypanosomiasis and nagana in cattle and constitute a serious health problem for people and livestock in 36 countries of sub-Saharan Africa. *Trypanosoma brucei* exists in the mammalian host as the bloodstream form trypanastigote and in the midgut of the tsetse fly vector (*Glossina* sp.) as the procyclic form. The bloodstream form of the parasite in the mammalian host is covered by a glycosylphosphatidylinositol (GPI)-anchored coat of 5 × 10⁶ variant surface glycoprotein (VSGs) homodimers and it evades the immune system by periodically replacing the existing VSG coat by a different one, a phenomenon known as antigenic variation (Cross 1996). Depending on the VSG variant GPI anchors contain side chains of 0–6 Gal residues (Ferguson et al. 1988) and between 1 and 3 N-linked glycans. The latter can be of the conventional oligomannose, paucimannose or complex types (Zamze et al. 1990, 1991; Jones et al. 2005; Manthri et al. 2008; Izquierdo, Schulz, et al. 2009).

When bloodstream form parasites are ingested by the tsetse fly, they differentiate to the procyclic form in the insect midgut. During
this process, the VSG coat is replaced by a mix of GPI-anchored pro-
cyclins, a non-GPI surface coat (Guther et al. 2006) as well as free GPIs
(Lillico et al. 2003; Vassella et al. 2003; Nagamune et al. 2004; Roper et al. 2005). Procyclins are characterized by internal dipeptide (EP) or
pentapeptide (GPEET) repeats which confer a rod-like structure to the protein (Roditin et al. 1989; Treumann et al. 1997).

Trypanosoma brucei strain 427, the strain used in this study, con-
tains (per diploid genome): two copies of the GPEET1 gene encoding six
GPEET repeats; one copy each of the EP1-1 and EP1-2 genes, en-
coding EP1 procyclins with 30 and 25 EP repeats, respectively; two
copies of the EP2-1 gene, encoding EP2 procyclin with 25 EP repeats;
and two copies of the EP3-1 gene, encoding EP3 procyclin with 22 EP
repeats (Acosta-Serrano et al. 1999). The EP1 and EP3 procyclins con-
tain a single N-glycosylation site, occupied exclusively by a conven-
tional triantennary oligosaccharide Man$_3$GlcNAc$_2$ oligosaccharide, at
the N-terminal side of the EP repeat domain (Treumann et al.
1997; Acosta-Serrano et al. 1999). Either EP2 or GPEET procyclins
are N-glycosylated and GPEET1 procyclin is phosphorylated on six of
seven Thr residues (Mehlert et al. 1999; Schlaeppi et al. 2003). Both
GPEET and EP procyclins contain indistinguishable GPI mem-
brane anchors. These are the largest and most complex anchors
known and they are characterized by the presence of large
poly-disperse-branched N-acetyllactosamine (Galβ1-4GlcNaC) and
lacto-N-biose (Galβ1-3GlcNaC)-containing side chains that can be
capped with α2-3-linked sialic acid residues (Ferguson et al. 1993;
Treumann et al. 1997). Sialic acids are transferred from serum sialo-
glycoconjugates to terminal β-galactose residues by the action of a cell
surface trans-sialidase enzyme (Engstler et al. 1993; Pontes de Carval-
ho et al. 1993; Montagna et al. 2002, 2006) and trans-sialylation of
surface components plays a role in the successful colonization of the
tsetse fly (Nagamune et al. 2004). In addition, it has been postulated
that the branched side chains of the anchor form a dense glyocalyx
that contributes to the protective function of the coat against digestive
enzymes in the fly midgut (Acosta-Serrano et al. 2001).

As well as the aforementioned major GPI-anchored surface mole-
cules, T. brucei expresses numerous other GPI-anchored and trans-
membrane glycoproteins at the cell surface, in the flagellar pocket and
in the intracellular endosomal–lysosomal system, some of which are
lifecycle stage- or display lifecycle stage-specific glycosylation dif-
fences. For example, the transmembrane invariant surface glycopro-
teins ISG65 and ISG75 (Ziegelbauer and Overath 1992) and the
GPI-anchored flagellar pocket ESA66/ESA67 heterodimeric transfer-
in receptors (Steverding et al. 1995; Steverding 2000; Mehlert and
Ferguson 2007; Mehlert et al. 2012) are specific to the bloodstream
lifecycle stage while the major lysosomal glycoprotein is common to
bloodstream and procyclic stages but contains complex N-glycans
only in the bloodstream stage (Kelley et al. 1995). This control of
stage-specific glycosylation probably resides primarily at the level of

The survival strategies of protozoan parasites frequently involve the
participation of glycoconjugates. Trypanosoma brucei expresses many
glycoproteins containing Gal and GlcNaC, including glycoproteins with
novel bloodstream form-specific giant poly-N-acetyllactosamine
(poly-LacNaC) containing N-linked glycans (Mehlert, Richardson,
et al. 1998; Mehlert, Zitzmann, et al. 1998; Atri et al. 2003). The cre-
ation of UDP-glucose 4′-epimerase (TbGde) conditional null mutants
showed that this gene, and hence UDP-Gal, is essential for the survival
of the parasite in both the bloodstream and procyclic life stages (Roper et al. 2002; Roper et al. 2005; Urbania et al. 2006). Similarly, the cre-
ation of UDP-GlcNaC pyrophosphorylase (TbUap) and glucosamine
6-phosphate N-acetyltransferase (TbGna1) conditional null mutants
has shown that UDP-GlcNaC is also essential for bloodstream form
T. brucei (Marino et al. 2011; Stokes et al. 2008). From these experi-
ments, it is possible to conclude that one or more of the UDP-
Gal- and UDP-GlcNaC-dependent glycosylation pathways are essential
to the parasite. This has provided the impetus to identify and charac-
terize the UDP-Gal- and UDP-GlcNaC-dependent glycosyltransferase (GT)
genes in the parasite. In a recent study, we mined the T. brucei
genome for GTs and found a family of 21 genes with predicted amino acid
sequences consistent with being UDP-sugar-dependent GTs.

The first of these genes to be studied were TbGT8, a UDP-GlcNaC:
βGal β1-3 GlcNac-transferase that modifies the complex GPI anchor
side chains of the procyclins (Izquierdo, Nakaniishi, et al. 2009) and
also elaborates complex N-glycans in bloodstream from parasites
(Nakaniishi et al. 2014), and TbGT11, a UDP-GlcNaC:Man β1-2
GlcNaC-transferase equivalent to GntI and involved in the synthesis
of complex N-glycan structures (Damerow et al. 2014).

In this work, we apply the methodology previously described for
the analysis and phenotyping of GT mutants of T. brucei (Izquierdo,
Nakaniishi, et al. 2009; Izquierdo et al. 2013), to biochemically char-
acterize another UDP-sugar-dependent GT, Tb927.2.3370 referred to
here as TbGT3.

Results
Creation and analysis of a bloodstream form TbGT3
conditional null mutant
We selected a putative GT (Tb927.2.3370) from T. brucei to determine
its activity by the creation and biochemical characterization of a condi-
tional null mutant under nonpermissive conditions. The gene encodes
a 377 amino acid protein, with a theoretical mass of 43.7 kDa. A semi-
quantiative RT-PCR analysis previously performed over 21 full-length
ORFs encoding putative UDP-Gal- or UDP-GlcNaC-dependent GTs in
the T. brucei genome showed that the gene was present in both life cycle
stages of the parasite, but more highly expressed in procyclic form try-
pansomes (Izquierdo, Nakaniishi, et al. 2009). This was confirmed in
a recent quantitative proteomics study, which indicated that TbGT3 was
expressed ∼2.5-fold higher in procyclic form trypansomes at the
protein level (Urbania et al. 2013).

The DXD sequence motif, common to many GTs (Wiggins and Munro 1998) and probably involved in binding a divalent cation, is
present in the predicted protein as DDD, between amino acids 206 and
208. The protein has a predicted N-terminal transmembrane domain
between residues 17–34 (Krogh et al. 2001) and is likely to be a
type II membrane protein, typical for Golgi apparatus GTs (Colley
1997). The Tb927.2.3370 gene and 5′ and 3′ untranslated flanking
sequences were amplified with a high-fidelity DNA polymerase from
T. brucei strain 427 genomic DNA. Four clones from four separate
PCR reactions were sequenced in both directions and the strain 427
sequence (Accession Number KF534011) was very similar to that
in the strain 927 genome database. In the ORF, there were four apparent
nucleotide polymorphisms and three of them produced amino acid
changes: his225 in place of Tyr225, Glm269 in place of Glu269 and
Arg301 in place of Gly301. The strain 427 gene and protein product
will be referred to from here as TbGT3 and TbGT3, respectively.

In order to analyze the function of the gene, we first created condi-
tional null mutants in the bloodstream and procyclic forms of the
parasite. A preliminary Southern blot analysis using a TbGT3 ORF
probe suggested that there is a single copy of the gene per haploid
genome (Supplementary data, Figure S1), which was also consistent
with BLAST search of the T. brucei genome database with the
Tb927.2.3370 nucleotide sequence. Both T. brucei TbGT3 alleles
were replaced in bloodstream form parasites and an ectopic inducible copy of \( T_{b}G{T}_{3} \) under tetracycline control was introduced. To generate the conditional null mutants, genetically modified bloodstream form strains from 427 \( T.\ brucei \) containing a T7-RNA polymerase and a tetracycline repressor under the control of a T7 promoter were used (Wirtz and Clayton 1995). This cell line will be referred to as “wild type” from here on.

The first \( T_{b}G{T}_{3} \) allele was replaced in the bloodstream form by homologous recombination following electroporation of the parasites in the presence of linear DNA containing the puromycin acetyltransferase (PAC) drug-resistance gene flanked by \( \sim -500 \) bp of \( T_{b}G{T}_{3} \). 5′- and 3′- untranslated region (UTR). Following selection with puromycin, a \( \Delta T_{b}G{T}_{3}:\text{PAC} \) mutant was selected and transformed with an ectopic, tetracycline-inducible, copy of \( T_{b}G{T}_{3} \), introduced into the ribosomal DNA locus under phleomycin selection (Wirtz et al. 1999). Then, maintaining tetracycline induction, the second endogenous allele was replaced by an hygromycin phosphotransferase (HYG) gene to yield the desired \( \Delta T_{b}G{T}_{3}:\text{PAC}/T_{b}G{T}_{3}^{\text{T}}/\Delta T_{b}G{T}_{3}:\text{HYG} \) bloodstream form mutant. Southern analysis confirmed that both endogenous \( T_{b}G{T}_{3} \) gene copies were absent from the mutant (Figure 1A). Northern blot showed that \( T_{b}G{T}_{3} \) mRNA transcripts were absent under nonpermissive conditions (i.e., with no tetracycline added to the media) (Figure 1B). This and the normal in vitro growth kinetics of the \( T_{b}G{T}_{3} \) mutant in the absence of tetracycline (data not shown) allows us to conclude that \( T_{b}G{T}_{3} \) encodes a nonessential gene in bloodstream form \( T.\ brucei \).

To observe any effects of the absence of \( T_{b}G{T}_{3} \) activity on \( T.\ brucei \) bloodstream-form glycosylation, wild-type and \( T_{b}G{T}_{3} \) conditional null variant surface glycoprotein 221 (VSG221) was purified in a soluble form (Cardoso de Almeida and Turner 1983; Ferguson et al. 1985) from cells grown in the presence and absence of tetracycline for 48 h. Mature wild-type sVSG221 glycoprotein contains two N-glycosylation sites, one at Asn296 occupied by paucimannose and small complex structures and another at Asn428 occupied by oligomannose structures (Zamze et al. 1991), and a highly galactosylated GPI anchor glycan (Mehlert, Richardson, et al. 1998; Mehlert, Zirnmann, et al. 1998). Thus, we use the glycosylation status of sVSG221 as a convenient reporter to assess any effects that a mutation has on the formation of several glycosidic linkages (Jones et al. 2005; Urbaniaik et al. 2006; Manthri et al. 2008; Izquierdo et al. 2012). Analysis of the glycoform pattern of sVSG221 from wild-type and \( T_{b}G{T}_{3} \) conditional null-mutant cells, grown in the presence and absence of tetracycline, by electrospray-mass spectrometry (ES-MS) did not reveal any alterations in its glycosylation pattern (data not shown).

In order to analyze the high-molecular-weight branched poly LacNac-containing N-glycans of bloodstream form \( T.\ brucei \) (Atrihi et al. 2003), we performed SDS/urea extraction of total glycoproteins from trypanosome ghosts (depleted of VSG) and western blotting with lectins. The extracts of wild-type and conditional cells before and after tetracycline induction were analyzed by ricin, which detects galactoses residues, and wheat germ agglutinin (WGA) blotting. In the case of the ricin blot (data not shown), the patterns were similar to each other and to previous ricin blots (Urbaniaik et al. 2006; Manthri et al. 2008; Stokes et al. 2008; Izquierdo, Nakanishi, et al. 2009). However, the absence of \( T_{b}G{T}_{3} \) activity due to nonpermissive conditions very slightly decreased the binding of WGA (Figure 1C, see high-molecular-weight species). WGA has complex-binding properties, including binding to GlcNAc\(B\)-Gal motifs (Muraki et al. 2002), which are characteristic of branched poly-N-acetyllactoaminoglycans previously described in blood stream trypanosomes. Thus, the differences observed suggest that the \( T_{b}G{T}_{3} \) producer may play a minor role in the biosynthesis of branched poly-LacNac structures in the bloodstream form of the parasite.

**Analysis of a procyclic form \( T_{b}G{T}_{3} \) conditional null mutant under nonpermissive conditions**

In order to investigate if a more dramatic phenotype could be observed in the procyclic form of the parasite, a \( T_{b}G{T}_{3} \) conditional null mutant was prepared in this stage of the parasite using cell line 29.13.6 (derived from \( T.\ brucei \) strain 427), that retain genomic T7-RNA polymerase and TETR genes in presence of G418 and hygromycin selection (Wirtz et al. 1999). The \( T_{b}G{T}_{3} \) alleles were replaced using the same targeting vectors used in the bloodstream form of the parasite, but containing PAC and blasticidin S deaminase (BSD) drug-resistance cassettes and an ectopic, tetracycline-inducible, copy of \( T_{b}G{T}_{3} \), introduced into the ribosomal DNA locus under phleomycin selection increasing the number of \( T_{b}G{T}_{3} \) genomic copies (Wirtz et al. 1999). The glycosylation phenotype was assessed in this \( T_{b}G{T}_{3} \) procyclic form conditional null mutant, \( \Delta T_{b}G{T}_{3}:\text{PAC}/T_{b}G{T}_{3}^{\text{T}}/\Delta T_{b}G{T}_{3}:\text{BSD} \), after confirmation by Southern blot that both \( T_{b}G{T}_{3} \) gene endogenous copies were absent in the mutant (Figure 2A). As in the case of the bloodstream form, procyclic form conditional null mutants were able to grow normally in vitro without the presence of tetracycline in the media, showing that the gene is also nonessential in the procyclic form of the parasite. Furthermore, mutants grown in nonpermissive conditions were able to colonize the tsetse midgut.
although the infectivity was marginally reduced compared with that of the wild type (Figure 2B). To assess the glycosylation phenotype of procyclic form parasites, procyclins from wild-type and conditional null mutant grown in the absence of tetracycline were extracted (Ferguson et al. 1993; Izquierdo et al. 2013) and analyzed by MS/MS and MALDI-ToF after aq. HF dephosphorylation with and without mild acid treatment (Acosta-Serrano et al. 1999; Mehlert et al. 1999). The dual aq. HF dephosphorylation and periodate-Schiff staining SDs-PAGE gel of extracted procyclins from wild type (lane 1) and TbGT3 mutant cells grown without tetracycline (lane 2) or with tetracycline (lane 3).

Characterization of the changes in the procyclin GPI anchor side chain in the TbGT3 conditional null mutant under nonpermissive conditions

In order to analyze the specific changes in procyclin GPI anchor side chain structure induced by the lack of TbGT3 expression, procyclins were isolated from wild-type and TbGT3 conditional null mutant grown in the absence of tetracycline. GPI anchor glycans were released from the procyclin peptide and the glycosyl-phosphatidic acid lipid component of the PI moiety by aq. HF dephosphorylation (a procedure that also removes some sialic acid residues). The released GPI glycans were subsequently permethylated, a procedure that removes the fatty acid from the inositol ring, methylates all free hydroxyl groups and converts the amine group of the glucosamine residue to a positively charged quaternary amine (Baldwin 2005; Mehlert and Ferguson 2007; Izquierdo, Nakanishi, et al. 2009; Nett et al. 2010). Analysis of the permethylated GPI glycan fractions by positive ion MALDI-ToF produced complex spectra (Figure 4). The [M]+ molecular ions in the spectra can be accounted for by assuming core structures of Hex₆, HexN(Me₃)+Ino substituted with between zero and eight HexHexNAc units and with the presence of sialic acid residues in some cases (Figure 4A). Although not a quantitative technique, the glycan species from the TbGT3 conditional null mutant grown under nonpermissive conditions appear to be significantly smaller (Figure 4B). Series from Hex,HexN(Me₃)+Ino, most probably from HexHexN(Me₃)+Ino losing a terminal galactose residue, can also be observed in panels A and B (see the open circles plus arrows).

The same permethylated GPI glycan samples were also analyzed by positive ion ESI-MS and doubly charged [M+Na]+ ions of (HexHexNAc)HexHexN(Me₃)+Ino species were observed at m/z 994.51. Fragmentation of these ions (MS²) produced an intense singly charged product ion at m/z 1696.81, arising from elimination of the inositol residue and quaternary amine group (Supplementary data Figure S2), which on further fragmentation (MS³) produced the product ion spectra shown in Figure 5. The spectra show differences in the compositions of the isobaric permethylated glycans between the two samples. The wild-type sample contains two principle components, (HexNAc)HexHexN(Me₃)+Ino and (HexHexNAc)HexHexN(Me₃)+Ino (Figure 5A), whereas the TbGT3 mutant only contains the (HexNAc)HexHexN(Me₃)+Ino species (Figure 5B). These data strongly suggest that TbGT3 encodes a Gal transferase that acts on the GPI anchor side chain to add a Gal residue to the non-reducing-terminal GlcNAc residue.

The same permethylated GPI glycan fractions were subjected to acid hydrolysis, reduction and acetylation to provide partially methylated alditol acetate derivatives (PMAAs) that were analyzed by GC–MS to provide a methylation linkage analysis (Figure 6; Supplementary data, Table S1). Normalizing the PMAA signals to the 6-O-substituted Man residue of the conserved Man₆Glcn-Ino core, we can see that the TbGT3 mutant GPI glycan is deficient in 3-O-substituted Gal residues and does not contain any 3-O-substituted GlcNAc residues, whereas wild-type GPI glycans contain both 4- and 3-O-substituted GlcNAc residues. Thus, the methylation linkage data suggest that TbGT3 is a β1-3 Gal-transferase that acts on one of the GlcNAc residues of the GPI anchor side chain. Consistent with this, some non-reducing-terminal GlcNAc (t-GlcNAc) is also apparent in the methylation linkage analysis (Figure 6B). Interestingly, these are not the only changes of PMAAs relative to 6-O-substituted Man; there are significant increases in the repertoire of procyclin proteins and the size of their N-glycans remained unchanged.
4-O-substituted GlcNAc and 3,6-di-O-substituted Gal residues, suggesting that there may be some compensatory mechanism at work in the absence of TbGT3 activity. This could include, for example, the modification of some of the βGlcNAc normally used as the acceptor substrate by TbGT3 by a β1-4 Gal-transferase.

Discussion

The survival strategies of protozoan parasites frequently involve the participation of glycoconjugates. In the particular case of T. brucei, several precursors required for the biosynthesis of these glycoconjugates such as UDP-Gal and UDP-GlcNAc (Roper et al. 2002, 2005; Stokes et al. 2008; Marino et al. 2011) are essential for the survival of the parasite in the bloodstream and/or procyclic form life stages. This prompted us to mine the T. brucei genome for UDP-sugar-dependent GTs and to investigate their essentiality, function and specificity by the creation of null or conditional null mutants. The fact that the TbGT3 conditional null-mutant cell lines grow normally in vitro in the absence of tetracycline in both the procyclic and the bloodstream form of the parasite, show that TbGT3 is non-essential for the survival of T. brucei. Similarly, experiments carried out in mice and tsetse flies indicate that the gene is nonessential for the survival of the parasite in vivo, though of course these types of experiment generally fail to pick up subtle fitness traits that can be sufficient to maintain the genes in nature.

Using a well-established workflow for the functional and biochemical characterization of T. brucei GTs (Izquierdo, Nakanishi, et al. 2009; Izquierdo et al. 2013), we designate TbGT3 as primarily a
GPI side chain modifying UDP-Gal: βGlcNAc β1-3 Gal-transferase. However, like TbGT8, which is both a GPI side-chain modifying UDP-GlcNAc βGal β1-3 GlcNAc-transferase (Izquierdo, Nakanishi, et al. 2009) and involved in large complex N-glycan synthesis in bloodstream form parasites (Nakanishi et al. 2014), TbGT3 is likely also to be involved in some way in the synthesis of large complex N-glycans in the bloodstream form of the parasite (Atrih et al. 2005; Izquierdo, Nakanishi, et al. 2009).

With respect to the GPI anchor modifying activity of TbGT3, we postulate that it acts on the product of TbGT8, either directly or after the action of an unknown α1-3 Gal-transferase (Figure 7).

**Materials and methods**

**Cultivation of trypanosomes**

Bloodstream form *T. brucei* genetically modified to express T7 polymerase and the tetracycline repressor protein were cultivated in HMI-9 medium containing 2.5 μg mL⁻¹ G418 at 37°C in a 5% CO₂ incubator as described in Wirtz et al. (1999). *Trypanosoma brucei* procyclic form parasites that maintain T7 polymerase and tetracycline repressor protein under G418 and hygromycin selection (cell line 29:13) (Wirtz et al. 1999) were cultured in SDM-79 medium at 28°C.

**DNA isolation and manipulation**

Plasmid DNA was purified from *Escherichia coli* (DH5α) using QiaGene Miniprep or Maxiprep kits, as appropriate. Gel extraction was performed using Qiaquick kits. Custom oligonucleotides were obtained from the Dundee University oligonucleotide facility. *Trypanosoma brucei* genomic DNA was isolated from ~2 × 10⁸ bloodstream form cells or from 1 × 10⁹ procyclic cells using DNAzol (Helena Biosciences).

**Generation of constructs**

The 413 bp 5'- and 401 bp 3'-UTR sequences next to the Tb927.2.3370 ORF were PCR-amplified from genomic DNA using Platinum Taq DNA polymerase HiFi (Invitrogen) with 5'-argGCCCG CGCCGtagaaggaaagttgctagttggt3' and 5'-gttaaaactcagcgcgtcgaaggt tttcggacgtgtaacctaaataga3' and 5'-gagcgttcgtagtttgaagccggatattagg ctcaattttgaag -3' and 5'-gttGGCCGGCGC gtaaacacacagaga aaacaca-3' as forward and reverse primers, respectively. The two...
PCR products were used together in a further PCR reaction to yield a product containing the 5′-UTR linked to the 3′-UTR by a short HindIII and BamHI cloning site (underlined) and NotI restriction sites at each end (capital letters). The PCR product was cloned into the NotI site of the pGEM-5Zf(+) vector (Promega) and the HYG and PAC drug-resistance genes were introduced into the targeting vector via the HindIII and BamHI cloning sites. For re-expression of *Tb927.2.3370* the ORF was PCR amplified from genomic DNA using Platinum Taq DNA polymerase HiFi with 5′-cgcAAGCTT atggtgtcgaagggtttac-3′ and 5′-cgcGGATCCtcagcatttcccagcagc-3′ and cloned into the pLew100 vector (Wirtz et al. 1999) cut with HindIII and BamHI, to generate pLewGT3. This plasmid was then digested with NotI, and purified for transfection.

Transformation of bloodstream and procyclic form *T. brucei*

Constructs for gene replacement and ectopic expression were purified using the Qiagen Maxiprep kit, digested with NotI to linearize, precipitated and washed twice with 70% ethanol and re-dissolved in sterile water. Cell culture and transformation were carried out as previously described (Wirtz et al. 1999; Milne et al. 2001).

Southern and Northern blotting

Aliquots of genomic DNA isolated from *T. brucei* cultures were digested with various restriction enzymes. Fluorescein-labelled probes were generated using the CDP-star random prime labelling kit (Gene Images); 250 ng of template was used in a reaction volume of 50 μL and incubated for 90 min. Aliquots of 5 μL were used for each Southern blot experiment.

Total RNA for Northern blots was prepared using Qiagen RNeasy Midi kits. Samples of RNA (5 μg) were run on formaldehyde-agarose gels and transferred to Hybond N nylon membrane (Amersham Biosciences) for hybridization with [α-32P]-dCTP-labeled *T. brucei* Tb927.2.3370 probe (Stratagene, Prime-It RmT random primer labelling kit). The control β-tubulin probe was used after the Tb927.2.3370 probe had decayed.

Tsetse fly infections

Tsetse fly infections were carried out as described in Guther et al. (2006). Pupae of *Glossina morsitans morsitans* were obtained from Institute of Zoology, Slovak Academy of Science (Bratislava, Slovakia) and maintained at the WCMP tsetse fly facility, University of Glasgow. Newly hatched (teneral) flies were fed with an infected bloodmeal, which consisted of 10^7 parasites mixed with washed defibrinated horse blood (containing 10% fetal bovine serum). Infected flies were fed with bloodmeals every 2–3 days. After 2 weeks or 3 weeks, midguts were isolated from infected flies and disrupted by mechanical force in cold SDM-79 containing 10% fetal bovine serum. Isolated parasites from individual midguts were kept on ice until counted on an hemocytometer.
Puriﬁcation of procyclins

Procyclins (both GPEET and EP forms) were puriﬁed from 10^11 freeze-dried trypanosomes by organic solvent extraction followed by octyl-Sepharose chromatography (Amersham Pharmacia Biotech) (Ferguson et al. 1993; Treumann et al. 1997; Mehlert et al. 1999). For some speciﬁc procedures, such as SDS–PAGE and periodate-Schiff stain, procyclins were extracted from batches of ∼1×10^8 cells and were run directly with the extracts without prior puriﬁcation by octyl-Sepharose chromatography.

Analysis by MALDI-ToF MS

Approximately 250 pmol of octyl-Sepharose-puriﬁed procyclins determined by GC–MS (Ferguson 1994) was dried and treated with 50 μL of ice-cold 50% aqueous hydrogen ﬂuoride for 24 h at 0°C to cleave the GPI anchor ethanolamine-phosphate bond. Some preparations were further treated with 50 μL of 40 mM trifluoroacetic acid, 100°C for 20 min to cleave Asp-Pro bonds and remove N-glycosylated N termini (Acosta-Serrano et al. 1999). The samples were dried and re-dissolved in 5 μL of 0.1% trifluoroacetic acid. Aliquots (1 μL) of each sample were mixed with 1 μL of 10 mg mL^{-1} sinapinic acid in 50% acetonitrile, 0.1% trifluoroacetic acid and analyzed by positive ion MALDI-ToF. Data collection was in linear mode on a Voyager-DE STR instrument. The accelerating voltage was 25,000 V, and grid voltage was set at 94% with an extraction time delay of 700 ns. Data were collected manually at 500 shots/spectrum with the laser intensity set at 2500. The analysis of the permethylated GPI glycans, the instrument was set in reflectron mode, positive ion and a matrix of 2,5-dihydroxybenzoic acid (15 mg mL^{-1} in 0.5% TFA) was used.

Permethylation and ES-MS of GPI glycans

Samples were dried and permethylated by the sodium hydroxide and methyl iodide method, as described in Ferguson (1994). The permethylated glycans were dissolved in 50 μL of 80% acetonitrile, and aliquots (5 μL) were dried and recovered in 80% acetonitrile, 0.5 mM sodium acetate before loading into nanotips (Micromass type F) for positive ion ES-MS, ES-MS2 and ES-MS3 on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientiﬁc). Source and capillary voltages were 0.63 kV and 48 V, respectively, and the collision energy was 17–19%.

Methylation linkage analysis by GC–MS

The remainder of the permethylated glycan samples were subjected to acid hydrolysis, NaB2H4 reduction and acetylation (to yield PMAAs), and analyzed by GC–MS as described in Ferguson (1994). The PMAAs were analyzed on an Agilent 6890 N GC–MS system ﬁtted with an HP-5 column.

Lectin blotting of T. brucei cell extracts

Bloodstream form T. brucei cells were washed twice in trypanosome dilution buffer (5 mM KCl, 80 mM NaCl, 1 mM MgSO4, 20 mM Na2HPO4, 2 mM NaH2PO4, 20 mM glucose, pH 7.4), solubilized
with 2% SDS and 4 M urea, subjected to SDS–PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were incubated for 1 h with biotin-conjugated ricin (Vector Labs) diluted 1/3000, with or without inhibitors (10 mg mL galactose and 10 mg mL lactose) or 1 h with biotin-conjugated WGA (Vector Laboratories) diluted 1/2000, with or without inhibitor (1/10 Chitin hyllosytlate). Membranes were then incubated for 1 h with extravidin–HRP diluted 1/10000, and developed with ECL (Amersham Biosciences) according to the manufacturer’s instructions.

**Supplementary data**

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

**Conflict of interest statement**

None declared.

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**Abbreviations**

BSD, blastidicin S deaminase; ES-MS, electrospray-mass spectrometry; GPI, glycosylphosphatidylinositol; GT, glycosyltransferase; HYG, hygromycin phosphotransferase; PMAAs, partially methylated alditol acetate derivatives; PAC, puromycin acetyltransferase; t-GlcNAc, terminal GlcNAc; UTR, untranslated region; VSGs, variant surface glycoprotein; VSG221, variant surface glycoprotein 221; WGA, wheat germ agglutinin.

**References**


Guther ML, Lee S, Tetley L, Acosta-Serrano A, Ferguson MA. 2006. GPI-anchored proteins and free GPI glycolipids of procyclic form Trypanosoma brucei are nonessential for growth, are required for colonization of the tsetse fly, and are not the only components of the surface coat. *Mol Biol Cell* 17:5263–5274.
