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

Luis Izquierdo2,3, Alvaro Acosta-Serrano4,5, Angela Mehlert2, and Michael AJ Ferguson2,1

2Division of Biological Chemistry and Drug Discovery, The College of Life Sciences, University of Dundee, Dundee DD1 5EH, UK, 3Barcelona Centre for International Health Research, CRESIB, Hospital Clinic-Universitat de Barcelona, Barcelona 08036, Spain, 4Department of Parasitology, and 5Department of Vector Biology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK

1To whom correspondence should be addressed: Tel: +44 1382 384219; Fax: +44 1382 322558; e-mail: m.a.j.ferguson@dundee.ac.uk

Received 11 October 2014; Revised 26 November 2014; Accepted 26 November 2014

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-GlcNAc-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 trypomastigote 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 $5 \times 10^9$ 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 (Guthrie 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 (Roditi 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 procyclin with 30 and 25 EP repeats, respectively; two
copies of the EP1-1 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 procyclin con-
tain a single N-glycosylation site, occupied exclusively by a conven-
tional triantennary oligomannose Man5GlcNAc2 oligosaccharide,
at the N-terminal side of the EP repeat domain (Treumann et al.
1997; Acosta-Serrano et al. 1999). Either EP2 or GPEET procyclin
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 procyclin 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 (Galβ1-3GalNAc) 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-
fersences. For example, the transmembrane invariant surface glycopro-
teins ISG65 and ISG75 (Ziegelbauer and Overath 1992) and the
GPI-anchored flagellar pocket ESA66/ESA7 heterodimeric transfer-
rin receptors (Steverding et al. 1993; Steverding 2008; Mehler and
Ferguson 2007; Mehler 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
oligosaccharyltransferase expression (Izquierdo, Schulz, et al. 2009).

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 (Mehler, Richardson,
et al. 1998; Mehler, Zittmann, et al. 1998; Atri et al. 2003). The cre-
ation of UDP-glucose 4′-epimerase (TbGalE) 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; Urbaniai et al. 2006). Similarly, the cre-
ation of UDP-GlcNAc pyrophosphorylase (TbUAP) and glucosamine
6-phosphate N-acetylttransferase (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 characte-
rize 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
(Nakanishi 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-
quantitative 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-
panosomes (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 trypanosomes at the
protein level (Urbaniai 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 do-
main 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 KF554011) was very similar to that in
the strain 927 genome database. In the ORF, there were four apparent
sequence changes: his225 in place of Tyr225, Gln269 in place of Glu269 and
Arg301 in place of Gly301. The strain 427 gene and protein product
was created conditionally null mutant under nonpermissive conditions. The gene encodes a
377 amino acid protein, with a theoretical mass of 43.7 kDa. A semi-
quantitative 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-
panosomes (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 trypanosomes at the
protein level (Urbaniai 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 do-
main 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 KF554011) 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, Gln269 in place of Glu269 and
Arg301 in place of Gly301. The strain 427 gene and protein product
was created conditionally 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 *TbGT3* 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 *TbGT3* 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 ~500 bp of *TbGT3* 5'- and 3'-untranslated region (UTR). Following selection with puromycin, a Δ*TbGT3::PAC* mutant was selected and transformed with an ectopic, tetracycline-inducible, copy of *TbGT3*, 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 Δ*TbGT3::PAC/TbGT3*Δ::Δ*TbGT3::HYG* bloodstream form mutant. Southern analysis confirmed that both endogenous *TbGT3* gene copies were absent from the mutant (Figure 1A). Northern blot showed that *TbGT3* 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 *TbGT3* mutant in the absence of tetracycline (data not shown) allows us to conclude that *TbGT3* encodes a nonessential gene in bloodstream form *T. brucei*.

To observe any effects of the absence of *TbGT3* activity on *T. brucei* bloodstream-form glycosylation, wild-type and *TbGT3* conditional null-mutant 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 glycans (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; Urbaniak et al. 2006; Manthri et al. 2008; Izquierdo et al. 2012). Analysis of the glycoform pattern of sVSG221 from wild-type and *TbGT3* 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. 2005), 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 galactose 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 (Urbaniak et al. 2006; Manthri et al. 2008; Stokes et al. 2008; Izquierdo, Nakanishi, et al. 2009). However, the absence of *TbGT3* 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 GlcNAcβ1-6Gal motifs (Muraki et al. 2002), which are characteristic of branched poly-N-acetyllactoamino-glycans previously described in blood stream trypanosomes. Thus, the differences observed suggest that the *TbGT3* 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 *TbGT3* 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 *TbGT3* 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 *TbGT3* 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 *TbGT3*, introduced into the ribosomal DNA locus under phleomycin selection increasing the number of *TbGT3* genomic copies (Wirtz et al. 1999). The glycosylation phenotype was assessed in this *TbGT3* procyclic form conditional null mutant, Δ*TbGT3::PAC/TbGT3*Δ::Δ*TbGT3::BSD*, after confirmation by Southern blot that both *TbGT3* 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,
fragments that are characteristic of the procyclin types being EP-repeats of the EP-procyclins. This yields N-glycosylated peptide HF dephosphorylation with mild acid treatment cleaves off the GPI extracted (Ferguson et al. 1993; Izquierdo et al. 2013) and analyzed by null mutant grown in the absence or presence of tetracyclin were ex-
ted terms generated by GPI anchor removal alone still contain the
this case, we can see ions corresponding to the EP3 and EP1-2 protein
of procyclin proteins and the size of their N-glycans remained unchanged.

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 tetracyclin. GPI anchor glycans were released from the procyclin peptide and the lyso-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; Mehler 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 Hex₄, Hex(N(Me₃))N(Ino substituted with between zero and eight HexHex-
NAc 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,Hex(N(Me₃))Ino, most probably from Hex,Hex(N(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 (Hex-
NAc)Hex,Hex(N(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)Hex,Hex(N(Me₃))Ino and (HexHexNAc)Hex,Hex(N(Me₃))Ino (Figure 5A), whereas the TbGT3 mutant only contains the (HexNAc)Hex,Hex(N(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 GkNAc 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 glycan contains both 4- and 3-O-substituted GlcNAc. 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

![Figure 2](tbrucei.png)

**Fig. 2.** TbGT3 conditional null-mutant procyclic cells grown in the absence of tetracycline are infective to tsetse flies and express smaller procyclin molecules than the wild type. (A) Southern blot of genomic DNA from procyclic form wild type (lane 1), wild-type + TbGT3 (lane 2) and TbGT3 conditional null mutant (ΔTbGT3::PAC-TbGT3(ΔTbGT3::HYG)) (lane 3) digested with HindIII and probed with TbGT3 ORF. The arrowheads indicate the bands corresponding to the endogenous (End) and ectopic (Ect) copies of the TbGT3 gene. (B) Teneral tsetse flies were fed with bloodmeals containing either wild-type or GT3 null mutants in the absence of tetracycline (tet). After 22 days, the flies (40 in each group) were dissected and midgut infections scored as heavy (black; >100 trypanosomes/field), intermediate (dark gray; 20–100 trypanosomes/field), weak (light gray; 1–19 trypanosomes/field) or no (detectable cells). (C) Periodate-Schiff stained 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).
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 and the blood stream 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 cultured 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 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 Qiagen 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'-agtGCGG CGCCGcagaaaggttctgagct-3' and 5'-gtaaaaactcaggacgccaagct tgtccgacgctataacaaagaa-3' and 5'-gaggctcattgtaaataagctgcttc-tttaggctcaattgtaaag -3' and 5'-gatGCCGCCGC gtaacacacacagaa aacaaca-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′-cgcGGATCCtcagcatttcccacgagc-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 labeling 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 107 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, NaBH₄ 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
supported by Wellcome Trust Strategic Award 097945 and we thank Douglas Lamont for assistance. Funding to pay the Open Access publication charges for this article was provided by The Wellcome Trust.

Abbreviations

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

References

Acosta-Serrano A, Cole RN, Mehler A, Lee MG, Ferguson MA, Englund PT. 1999. The procyclin repertoire of Trypanosoma brucei. Identification and structural characterization of the Glu-Pro-rich polypeptides. J Biol Chem 274:29763–29771.

Acosta-Serrano A, Vassella E, Liniger M, Kunz Renggli C, Brun R, Roditi I, Englund PT. 2001. The surface coat of procyclic Trypanosoma brucei: programmed expression and proteolytic cleavage of procyclin in the tsetse fly. Proc Natl Acad Sci USA. 98:1513–1518.

Atrih A, Richardson JM, Prescott AR, Ferguson MA. 2005. Trypanosoma brucei glycoproteins contain novel giant poly-N-acetyllactosamine carbohydrate chains. J Biol Chem 280:865–871.

Baldwin MA. 2005. Analysis of glycosylphosphatidylinositol protein anchors: The prion protein. Methods Enzymol. 405:172–187.

Cardoso de Almeida ML, Turner MJ. 1983. The membrane form of variant surface glycoproteins of Trypanosoma brucei. Nature. 302:349–352.

Colley KJ. 1997. Golgi localization of glycosyltransferases: More questions than answers. Glycobiology. 7:1–13.

Cross GA. 1996. Antigenic variation in trypanosomes: Secrets surface slowly. BioEssays. 18:283–291.

Damerow M, Rodrigues JA, Wu D, Guther ML, Mehlert A, Ferguson MA. 2014. Identification and functional characterization of a highly divergent N-acetylgalcosaminyltransferase I (TbGnTI) in Trypanosoma brucei. J Biol Chem. 289:9328–9339.

Engert M, Reuter G, Schauer R. 1993. The developmentally regulated trans-sialidase from Trypanosoma brucei sialylates the procyclin glycoprotein. Mol Biochem Parasitol. 61:1–13.

Ferguson MA, Haldar K, Cross GA. 1985. Trypanosoma brucei variant surface glycoprotein has a sn-1,2-dimyristyl glycerol membrane anchor at its COOH terminus. J Biol Chem. 260:4964–4968.

Ferguson MA, Homans SW, Dwek RA, Radenacher TW. 1988. Glycosylphosphatidylinositol moiety that anchors Trypanosoma brucei variant surface glycoprotein to the membrane. Science. 239:753–759.

Ferguson MA, Murray P, Rutherford H, McConville MJ. 1993. A simple purification of procyclic acidic repetitive protein and demonstration of a sialylated glycosyl-phosphatidylinositol membrane anchor. Biochem J. 291(Pt 1):51–55.

Ferguson MAJ. 1994. Glycobiology: A Practical Approach. Oxford: IRL Press at Oxford University Press.

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. 17:5265–5274.

Izquierdo L, Guther ML, Ferguson MA. 2013. Creation and characterization of glycosyltransferase mutants of Trypanosoma brucei. Methods Mol Biol. 1022:249–275.
Izquierdo L, Mehler A, Ferguson MA. 2012. The lipid-linked oligosaccharide donor specificities of Trypanosoma brucei oligosaccharyltransferases. Glycobiology, 22:696–703.

Izquierdo L, Nakashima M, Mehler A, Machray G, Barton GJ, Ferguson MA. 2009. Identification of a glycosphingophosphatidylinositol anchor-modifying beta-1→3 N-acetylglucosaminyl transferase in Trypanosoma brucei. Mol Microbiol. 71:478–491.

Izquierdo L, Schulz BL, Rodrigues JA, Guther ML, Procter JB, Barton GJ, Aebi M, Ferguson MA. 2009. Distinct donor and acceptor specificities of Trypanosoma brucei oligosaccharyltransferases. EMBO J. 28:2650–2661.

Jones DC, Mehler A, Guther ML, Ferguson MA. 2005. Deletion of the glucosidase II gene in Trypanosoma brucei reveals novel N-glycosylation mechanisms in the biosynthesis of variant surface glycoprotein. J Biol Chem. 280:35928–35942.

Kelley RJ, Brickman MJ, Baljer AE. 1995. Processing and transport of a lysosomal membrane glycoprotein is developmentally regulated in African trypanosomes. Mol Biochem Parasitol. 74:167–178.

Krog A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane topology with a hidden Markov model: Application to complete genomes. J Mol Biol. 305:567–580.

Lillico S, Field MC, Blundell P, Coombs GH, Mottram JC. 2003. Essential roles of GPI-modifying galactosyltransferase in Trypanosoma brucei. Mol Biol Cell. 14:1182–1194.

Manthri S, Guther ML, Izquierdo L, Acosta-Serrano A, Ferguson MA. 2008. Deletion of the TbALG3 gene demonstrates site-specific N-glycosylation and N-glycan processing in Trypanosoma brucei. Glycobiology. 18:367–383.

Marino K, Guther ML, Wernimont AK, Qiu W, Hui R, Ferguson MA. 2011. The glycosylation of the variant surface glycoproteins and procyclic acidic glycoproteins in bloodstream form Trypanosoma brucei. Eukaryot Cell. 10:985–997.

Mehler A, Ferguson MA. 2007. Structure of the glycosphingophosphatidylinositol anchor of the Trypanosoma brucei transferrin receptor. Mol Biochem Parasitol. 151:220–223.

Mehler A, Richardson JM, Ferguson MA. 1998. Structure of the glycosphingophosphatidylinositol membrane anchor glycan of a class-2 variant surface glycoprotein from Trypanosoma brucei. J Mol Biol. 277:379–392.

Mehler A, Teumann A, Ferguson MA. 1999. Trypanosoma brucei GPI-activating protein of bloodstream form Trypanosoma brucei. Mol Biochem Parasitol. 98:291–296.

Mehler A, Wormald MR, Ferguson MA. 2012. Modeling of the N-glycosylated transferrin receptor suggests how transferrin binding can occur within the surface coat of Trypanosoma brucei. PLoS Pathog. 8:e1002618.

Mehler A, Zitzmann N, Richardson JM, Teumann A, Ferguson MA. 1998. The glycosylation of the variant surface glycoproteins and procyclic acidic repetitive proteins of Trypanosoma brucei. Mol Biochem Parasitol. 91:145–152.

Milne KG, Guther ML, Ferguson MA. 2001. Acyl-CoA binding protein is essential in bloodstream form Trypanosoma brucei. Mol Biochem Parasitol. 112:301–304.

Montagna G, Cremona ML, Paris G, Amaya MF, Buschiazzo A, Alzari PM, Frasch AC. 2002. The trans-sialidase from the African trypanosome Trypanosoma brucei. J Biol Chem. 277:392–397.

Montagna GN, Donelson JE, Frasch AC. 2006. Prooligolipid transfer in bloodstream form Trypanosoma brucei expresses separate sialidase and trans-sialidase enzymes on its surface membrane. J Biol Chem. 281:33949–33958.

Muraki M, Ishimura M, Harata K. 2002. Interactions of wheat-germ agglutinin with GlcNAc beta 1,6Gal sequence. Biochem Biophys Acta. 1569:10–20.

Nagamune K, Acosta-Serrano A, Uemura H, Brun R, Kunz-Renggli C, Maeda Y, Ferguson MA, Kinoshita T. 2004. Surface sialic acids taken from the host allow trypanosome survival in tsetse fly vectors. J Exp Med. 199:1445–1450.

Nakanishi M, Karasudani M, Shirasaki T, Hashida K, Hino M, Ferguson MA, Nomoto H. 2014. TbGT8 is a bifunctional glycosyltransferase that elaborates N-linked glycans on a protein phosphatase AcpP115 and a GPI-anchor modifying glycan in Trypanosoma brucei. Parasitol Int. 63:513–518.

Nett IR, Mehler A, Lamont D, Ferguson MA. 2010. Application of electrospray mass spectrometry to the structural determination of glycosphosphatidylinositol membrane anchors. Glycobiology. 20:576–585.

Pontes de Carvalho LC, Tomlinson S, Vanekerchhove F, Bijen EJ, Clarkson AB, Jiang MS, Hart GW, Nussenzeew V. 1993. Characterization of a novel trans-sialidase of Trypanosoma brucei procyclic tryptomastigotes and identification of procyclin as the main stalic acid acceptor. J Exp Med. 177:465–474.

Rodić I, Schwarz H, Pearson TW, Beecroft RP, Liu MK, Richardson JP, Buhring HJ, Pless J, Buloś R, Williams RO, et al. 1989. Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of Trypanosoma brucei. J Cell Biol. 108:737–746.

Roper JR, Guther ML, Macrae JL, Prescott AR, Hallyburton I, Acosta-Serrano A, Ferguson MA. 2005. The suppression of galactose metabolism in procyclic form Trypanosoma brucei causes cessation of cell growth and alters procyclin glycoprotein structure and copy number. J Biol Chem. 280:19728–19736.

Roper JR, Guther ML, Milne KG, Ferguson MA. 2002. Galactose metabolism is essential for the African sleeping sickness parasite Trypanosoma brucei. Proc Natl Acad Sci USA. 99:5884–5889.

Schleppe AC, Malherbe T, Butikofer P. 2003. Coordinate expression of GPIET procyclin and its membrane-associated kinase in Trypanosoma brucei procyclic forms. J Biol Chem. 278:49980–49987.

Steverding D. 2000. The transferrin receptor of Trypanosoma brucei. Parasitol Int. 48:191–198.

Steverding D, Stierhof YD, Fuchs H, Tauber R, Overath P. 1995. Transferrin-binding protein complex is the receptor for transferrin uptake in Trypanosoma brucei. J Cell Biol. 131:1173–1182.

Stokes MJ, Guther ML, Turnock DC, Prescott AR, Martin KL, Alphey MS, Ferguson MA. 2008. The synthesis of UDP-N-acetylglucosamine is essential for bloodstream form Trypanosoma brucei in vitro and in vivo and UDP-N-acetylglucosamine starvation reveals a hierarchy in parasite protein glycosylation. J Biol Chem. 283:16147–16161.

Treumann A, Zitzmann N, Hulsmeyer A, Prescott AR, Almond A, Sheehan J, Ferguson MA. 1997. Structural characterization of two forms of procyclic acidic repetitive protein expressed by procyclic forms of Trypanosoma brucei. J Mol Biol. 269:529–547.

Urbaniak MD, Martin DM, Ferguson MA. 2013. Global quantitative SILAC phosphoproteomics reveals differential phosphorylation is widespread between the procyclic and bloodstream form lifestyle stages of Trypanosoma brucei. J Proteome Res. 12:2233–2244.

Urbaniak MD, Turnock DC, Ferguson MA. 2006. Galactose starvation in a bloodstream form Trypanosoma brucei UDP-glucose 4-epimerase conditional null mutant. Eukaryot Cell. 5:1906–1915.

Vassella E, Butikofer P, Engstler M, Jelk J, Rodin J. 2003. Procyclin null mutants of Trypanosoma brucei express free glycosphingophosphatidylinositol on their surface. Mol Biol Cell. 14:1308–1318.

Wiggins CA, Munro S. 1998. Activity of the yeast MNN1 alpha-1,3-mannosyltransferase requires a motif conserved in many other families of glycosyltransferases. Proc Natl Acad Sci USA. 95:7945–7950.

Wirz E, Clayton C. 1995. Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. Science. 268:1179–1183.

Wirz E, Leal S, Ochatt C, Cross GA. 1999. A tightly regulated inducible expression system for conditional gene knock-out and dominant-negative genetics in Trypanosoma brucei. Mol Biochem Parasitol. 99:89–101.

Zamze SE, Ashford DA, Wooten EW, Rademacher TW, Dwek RA. 1991. Structural characterization of the asparagine-linked oligosaccharides from Trypanosoma brucei type II and type III variant surface glycoproteins. J Biol Chem. 266:20244–20261.

Zamze SE, Wooten EW, Ashford DA, Ferguson MA, Dwek RA, Rademacher TW. 1990. Characterisation of the asparagine-linked oligosaccharides from Trypanosoma brucei type I variant surface glycoproteins. Eur J Biochem. 187:657–663.

Ziegelbauer K, Overath P. 1992. Identification of invariant surface glycoproteins in the bloodstream stage of Trypanosoma brucei. J Biol Chem. 267:10791–10796.