THE SUPPRESSION OF GALACTOSE METABOLISM IN PROCYCLIC FORM
TRYPANOSOMA BRUCEI CAUSES CESSION OF CELL GROWTH AND ALTERS
PROCYCLIN GLYCOPROTEIN STRUCTURE AND COPY NUMBER*

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Running title: Galactose metabolism in procyclic T.brucei.

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Galactose metabolism is essential in bloodstream form T.brucei and is initiated by the
enzyme UDP-Glc 4'-epimerase. Here, we show that the parasite epimerase is a homodimer that
can interconvert UDP-Glc and UDP-Gal but not UDP-GlcNAc and UDP-GalNAc. The epimerase
was localized to the glycosomes by immunofluorescence microscopy and subcellular fractionation, suggesting a novel compartmentalization of galactose metabolism in this organism. The epimerase is encoded by the TbGALE gene and procyclic form T.brucei single-allele knockouts and conditional (tetracycline-inducible) null mutants were constructed. Under non-permissive conditions, conditional null mutant cultures ceased growth after 8 days and resumed growth after 15 days. The resumption of growth coincided with constitutive re-expression epimerase mRNA. These data show that galactose metabolism is essential for cell-growth in procyclic form T.brucei. The epimerase is required for glycoprotein galactosylation. The major procyclic form glycoproteins, the procyclins, were analysed in TbGALE single-allele knockouts and in the conditional null mutant after removal of tetracycline. The procyclins contain glycosylphosphatidylinositol membrane anchors with large poly-N-acetyl-lactosamine side-chains. The single allele knockouts exhibited 30% reduction in procyclin galactose content. This example of haploid insufficiency suggests that epimerase levels are close to limiting in this life-cycle stage. Similar analyses of the conditional null mutant 9 days after the removal of tetracycline showed that the procyclins were virtually galactose-free and greatly reduced in size. The parasites compensated, ultimately unsuccessfully, by expressing ten-fold more procyclin. The implications of these data with respect to the relative roles of procyclin polypeptide and carbohydrate are discussed.

The tsetse fly-transmitted protozoan parasite Trypanosoma brucei is responsible for human sleeping sickness and the cattle disease Nagana in sub-Saharan Africa. The organism undergoes a complex life cycle between the mammalian host and the insect vector. The bloodstream (trypomastigote) form of the parasite lives in the blood, lymph, interstitial fluids and, ultimately, the cerebrospinal fluid of the host. It avoids the host’s innate immune system through the expression of a dense monolayer of 10⁷ variant surface glycoprotein (VSG) molecules and it avoids specific immune responses through antigenic variation (1, 2). Thus, each parasite expresses only one of a repertoire of several hundred VSG genes at a time. The bloodstream form parasites exist as dividing ‘slender’ forms and non-dividing ‘stumpy’ forms that are pre-adapted for survival in the tsetse fly. Following ingestion in a blood meal, the stumpy trypomastigote form differentiates into the dividing procyclic form that colonises the tsetse midgut. The procyclic trypanosomes express a radically different cell surface coat made up about 3 x 10⁶ procyclin glycoproteins (3-6) and a smaller number of free glycoinositolphospholipids (GIPLs) (7-9). The procyclins are polyanionic, rod-like (6, 10), proteins encoded by four procyclin genes: GPEET, that encodes a protein with 5 or 6 Gly-Pro-Glu-Glu-Thr repeats and EP1, EP2 and EP3 that encode proteins with 18-30 Gly-Pro repeats (11). In T.brucei strain 427, used in this study, the parasites contain (per diploid genome) two copies of the GPEET1 gene encoding 6 Gly-Pro-Glu-Glu-Thr repeats, one copy

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each of the EP1-1 and EP1-2 genes, encoding EP1 procyclins with 30 and 25 Glu-Pro repeats, respectively, two copies of the EP2-1 gene, encoding EP2 procyclin with 25 Glu-Pro repeats and two copies of the EP3-1 gene, encoding EP3 procyclin with 22 Glu-Pro repeats (12). The EP1 and EP3 procyclins contain a single N-glycosylation site, occupied exclusively by a conventional Man$_3$GlcNAc$_2$ oligosaccharide, at the N-terminal side of the Glu-Pro repeat domain (6, 11). Whereas neither EP2 nor GPEET procyclin is N-glycosylated, GPEET1 procyclin is phosphorylated on six out of seven Thr residues (13-15). In culture, the procyclin expression profile depends on the carbon source (16) and metabolic state of the cells (17, 18) and in the tsetse fly there appears to be a program of procyclin expression such that GPEET procyclin is expressed early, giving way to EP1 and EP3 procyclin expression (16, 19). GPEET and EP procyclins are characterised by the presence of large poly-disperse branched poly-N-acetylglucosamine (Gal$_b$1-4GlcNAc) containing side-chains (with an average of about 8 to 12 repeats, depending on the preparation) that can terminate with α2-3 linked sialic acid residues (6, 20). Sialic acid is transferred from serum sialoglycoconjugates to terminal βGal residues by the action of a cell-surface trans-sialidase enzyme (21-23) and trans-sialylation of surface components plays a role in the successful colonisation of the tsetse fly (9). In vivo, the N-termini of the procyclins are removed by tsetse fly gut proteases and it is thought that the underlying (protease resistant) anionic repeat units and associated GPI anchor side-chains might protect the parasite from the approach tsetse fly gut hydrolases (19).

In this paper, we describe the nature and subcellular location of T. brucei UDP-Glc 4'-epimerase, an essential enzyme required for galactose metabolism in bloodstream form T. brucei (24), and show that it is also essential for the in vitro growth of procyclic form T. brucei. We also describe the changes in the cell surface molecular architecture of procyclic form T. brucei when they undergo partial and complete galactose starvation by genetic manipulation.

**EXPERIMENTAL PROCEDURES**

**Cell culture** - Procyclic form T. brucei cells (strain 29:13; a gift from G.A.M. Cross), referred to herein as wild type cells, were grown in SDM-79 medium (25) in the presence of 50 μg/ml hygromycin and 15 μg/ml G418 to maintain selection for the constitutively expressed T7RNAP and TETR genes, respectively. Bloodstream form T. brucei cells (strain 427, variant 221) were grown in HMI-9 medium (26) supplemented with 10% fetal calf serum at 37°C and 5% CO$_2$ as described in (24).

**Gel Filtration and Analytical Ultracentrifugation of Recombinant T. brucei UDP-Glc 4'-epimerase** - Bacterial expression and purification of recombinant epimerase have been described previously (24). Recombinant protein (100 μg at 1 mg/ml in phosphate-buffered saline (PBS)) was loaded onto a Superdex 200 HR30 HPLC column (Pharmacia Biotech). Proteins were eluted at 0.5 ml/min in PBS and monitored by absorbance at 280 nm. Fractions (2 ml) were collected and the presence of the epimerase in peak fractions was verified by SDS-PAGE. Molecular weight standards (cytochrome C 12.4 kDa, carbonic anhydrase 29 kDa, ovalbumin 43 kDa, bovine serum albumin 66 kDa, alcohol dehydrogenase 150 kDa, β-amylase 200 kDa, apoferritin 443 kDa and thyroglobulin 669 kDa) were run under the same conditions to calibrate the column.

Recombinant T. brucei epimerase (1 mg/ml PBS) was analysed by sedimentation velocity using a Beckman Optima XL-I analytical ultracentrifuge with an AN50-Ti rotor at 32,000 rpm at 20°C. Absorbance data (72 scans at 280 nm) were collected and analysed using the SEDFIT programme (27). The epimerase was assumed to be globular and its density was predicted from its amino acid composition.

**HPLC Assay of T. brucei UDP-Glc 4'-epimerase** - Epimerase reactions were performed with UDP-Gal, UDP-Glc, UDP-GalNAc or UDP-GlcNAc (0.8 mM final concentration) for 16 h at 37 C in 1 ml of 100 mM glycine buffer (pH 8.7), 1 mM β-NAD$^+$ containing 500 ng (2.95 mU) recombinant epimerase. Samples containing 10 nmol of sugar nucleotide from each reaction were loaded onto a Partisil P10 SAX column (25 cm x 0.46 cm; HiChrom) and analysed under conditions that separate UDP-hexoses (28). The column was eluted at 1.5 ml/min with 5 mM Na$_2$B$_4$O$_7$ for 2 min then with a linear gradient to 200 mM Na$_2$B$_4$O$_7$ over 40 min, held for 10 min. The eluate was monitored for absorbance at 262 nm. Standards of
NAD', UMP, UDP, UDP-Glc, UDP-Gal, UDP-GlcNAc, UDP-GalNAc (10 nmol) were run under the same conditions to calibrate the system.

**Fluorescence Microscopy** – Cultured procyclic form and bloodstream form *T. brucei* cells were harvested by centrifugation and washed and resuspended in ice-cold trypanosome dilution buffer (25 mM KCl, 400 mM NaCl, 5 mM MgSO₄, 100 mM Na₂HPO₄, 10 mM NaH₂PO₄ and 100 mM glucose) at a final concentration of 2 x 10⁷ cells/ml. The parasites were air-dried on cover slips (13 mm) at room temperature, fixed in 4% paraformaldehyde in trypanosome dilution buffer for 30 min at 4°C and permeabilized with 1% NP40 in phosphate-buffered saline (PBS). After washing four times with (PBS), fixed parasites were treated with 0.1% bovine serum albumin in PBS for 1 h to block non-specific binding and incubated for 1 h with pre-immune rabbit serum or rabbit antiserum (diluted 1/500 with 0.1% bovine serum albumin in PBS) raised against *T. brucei* glyceraldehyde phosphate dehydrogenase (GAPDH) or against recombinant *T. brucei* UDP-Glc 4'-epimerase (24). The coverslips were washed six times with PBS and incubated for 1 h with Alexa488 conjugated to anti-rabbit secondary antibody (Molecular Probes) diluted 1/500 with 0.1% bovine serum albumin in PBS. After six washes with PBS, specimens were mounted in Hydromount and single optical sections were collected on a Zeiss 510 META confocal microscope (alpha-Plan-Fluor x100).

**Subcellular Fractionation and Western Blotting** - Bloodstream form *T. brucei* (strain 427, variant 117) were isolated from infected rats and purified over DEAE-cellulose (29) and fractionated into large granular, small granular, microsomal and cytosolic fractions according to (30). Aliquots of these fractions (equivalent to 2.9 x 10⁸ cells) were subjected to SDS-PAGE on 10% NuPage (Invitrogen) gels and transferred to nitrocellulose. Western blotting was performed using rabbit polyclonal antibodies raised against recombinant UDP-Glc 4'-epimerase and affinity purified on immobilized recombinant UDP-Glc 4'-epimerase. Blots were incubated for 1 h at room temperature with 0.01 µg/ml antibody in Tris-buffered saline (pH7.4), 0.05% NP40, 2.5 mg/ml bovine serum albumen, washed three times with the same buffer, incubated 1 h with anti-rabbit conjugated to horse radish peroxidase (Scottish Antibody production Unit) diluted 1/5000 with the same buffer, washed three times and developed with ECL (Amersham) according to the manufacturers instructions.

**Generation of TbGALE Single-Allele Knockout and Conditional Null Mutant Procyclic form *T. brucei* Clones - Puromycin (PAC) and blasticidin (BSR) antibiotic resistance genes were cloned into *TbGALE*-targeted gene replacement plasmids as described in (24). The tetracycline-inducible expression plasmid (pLew100) containing the *TbGALE* ORF has been described previously (24). Linearised plasmids (10 µg) were introduced into mid-log (4-8 x 10⁶ cells/ml) procyclic cells, that had been washed and resuspended in cytomix (31) at 4 x 10⁷ cells/ml, by electroporation at 1.7 kV (3 pulses) using a BTX830 square-wave electroporator with a 630B shocking chamber and 0.4 cm gap cuvettes. Antibiotic selection (1 µg/ml for puromycin and 10 µg/ml for blasticidin) was added after overnight recovery in 10 ml SDM-79. From this stock, aliquots of 2 ml were plated in each of 4 wells of a 24-well plate and a series of doubling-dilutions in SDM-79 were made across the remaining rows. The remaining 2 ml was retained in a T10 flask and diluted 1:5 with SDM-79.

Epimerase expression from the inducible pLew100 vector was maintained by addition of 1 µg/ml tetracycline. To test if *TbGALE* was essential, conditional epimerase-null cells were washed three times in medium without tetracycline and cultures (with and without 1 µg/ml tetracycline) were inoculated at 1 x 10⁶ cells/ml. Cells were counted daily and cultures were diluted to 1 x 10⁶ cells/ml when densities were around 1 x 10⁷ cells/ml.

**Southern and Northern Blotting** - Genomic DNA for Southern blotting was prepared from 1-2 x 10⁸ cells using DNAzol (Helena Biosciences). *BSR, PAC, TbGALE* and *TbGALE* 5'-UTR probes were PCR amplified, purified, dUTP-fluorescein labelled by random priming (Gene Images Kit, Amersham Pharmacia) and used to probe PstI digests of 5 µg DNA.

Total RNA for Northern blots was prepared using Qiagen RNAeasy Midi kits. Samples of RNA (5 µg) were run on formaldehyde agarose gels and transferred to Hybond N nylon membrane (Amersham Pharmacia) for hybridisation with [α-³²P]dCTP-labelled *T. brucei TbGALE* probe (Stratagene, Prime-It RmT Random Primer labelling kit). The control β-tubulin probe was used after the *TbGALE* probe had decayed.
Procyclin Extraction and Analysis by MALDI-Tof Mass Spectrometry - Procyclins were extracted from batches of approximately $5 \times 10^7$ cells, as described in (12). Aliquots of 9% butanol-extracted procyclins (from the equivalent of $3 \times 10^7$ cells) were freeze dried and treated with 50 µl ice-cold 50% aqueous hydrogen fluoride (aq. HF) for 24 h at 0°C to cleave the GPI anchor ethanolamine-phosphate bond. After freeze drying, aliquots equivalent to $3 \times 10^6$ cells were further treated with 50 µl 40 mM trifluoroacetic acid (TFA), 100°C for 20 min, to cleave Asp-Pro bonds and remove N-glycosylated N-termini. The samples were dried and redissolved in 5 µl 0.1% TFA. Aliquots (0.5 µl) of each sample were mixed with 0.5 µl 20 mg/ml sinapinic acid in 70% acetonitrile, 0.1% TFA and analysed by negative-ion MALDI-Tof. Data collection was in linear mode on a Voyager-DE STR instrument. The accelerating voltage was 2500 V, grid voltage was set at 94%, with an extraction time delay of 700 nsec. Data were collected manually at 100 shots per spectrum, with laser intensity set at 250.

RESULTS

T. brucei UDP-Glc 4'-Epimerase is a Dimer in Aqueous Solution - The purification and kinetic properties of recombinant T. brucei epimerase has been described previously (24). To determine its oligomeric state, gel filtration and analytical ultracentrifugation of T. brucei recombinant epimerase were performed. The purified recombinant protein was applied to a calibrated Superdex 200 gel filtration column. A major protein and enzyme activity peak corresponding to 85 kDa was observed (data not shown). Analytical ultracentrifugation of the same material showed a main component of 79 kDa and a very minor component of 162 kDa (Fig. 1). These molecular masses are similar to those predicted for recombinant T. brucei UDP-Glc 4'-epimerase homodimer (87 kDa) and homotetramer (174 kDa), respectively. We conclude that T. brucei epimerase is predominantly a homodimer in aqueous solution, consistent with X-ray crystallographic data (34).

Substrate specificity of T. brucei UDP-Glc 4' - epimerase - The recombinant enzyme was incubated with either UDP-Glc, UDP-Gal, UDP-GlcNAc or UDP-GalNAc and the products were analysed by SAX-HPLC in the presence of borate ions; a chromatographic system that allows resolution of UDP-Glc from UDP-Gal and UDP-GlcNAc from UDP-GalNAc (28, 35). The HPLC profiles showed the inter-conversion of UDP-Glc and UDP-Gal (Fig. 2A, B). However, the T. brucei enzyme was unable to inter-convert UDP-GlcNAc and UDP-GalNAc (Fig. 2C, D).

T. brucei UDP-Glc 4'-Epimerase is Located in the Glycosome - Polyclonal rabbit antisera were raised to recombinant T. brucei epimerase and used to stain fixed bloodstream form and procyclic form T. brucei cells (Fig. 3A, B). The punctate staining pattern throughout the cell body is similar to that observed using polyclonal rabbit antibodies to the glycosome marker enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) (Fig. 3C, D). Pre-immune sera did not show immunoreactivity against fixed parasites (Fig. 3E, F). These data suggest that the T. brucei epimerase is located in the glycosomes in both life-cycle stages. This conclusion was supported...
by subcellular fractionation of bloodstream form parasites. The post-nuclear supernatant from mechanically disrupted trypanosomes was fractionated into a large granular (mitochondria-enriched), small granular (glycosome-enriched), microsomal (endoplasmic reticulum and Golgi apparatus-enriched) and cytosolic fractions by differential centrifugation according to (30). Aliquots of these fractions were analysed by SDS-PAGE and Western blotting with affinity purified anti-UDP-Glc 4’-epimerase (Fig. 3G). The results clearly show that the UDP-Glc 4’-epimerase is located predominantly in the small granular, glycosome-enriched, fraction.

UDP-Glc 4’-epimerase is Essential for the Growth of Procyclic Form T. brucei - It was possible to replace one TbGALE allele in procyclic form T. brucei by homologous recombination with either PAC or BSR. However, several attempts to replace the second allele with the complementary drug-resistance gene failed, suggesting that TbGALE might be an essential gene in procyclic form T. brucei. To investigate this, a conditional null mutant was created. A trypanosome cell line that constitutively expresses T7 RNA polymerase and the tetracycline repressor (TETR) protein under hygromycin and G418-neomycin selection, respectively, was used and an ectopic epimerase gene was introduced into the trypanosome rDNA locus using the pLew100 expression vector (36). Four conditional null mutant clones (TbGALEΔ, ΔTbGALE::PAC/ΔTbGALE::BSR) were obtained and Southern blots confirmed the replacement of both chromosomal TbGALE alleles with antibiotic resistance genes (Fig. 4).

To test whether the TbGALE gene is essential, conditional null mutant cells were washed three times in medium without tetracycline and cultures (with and without 1 µg/ml tetracycline) were inoculated. Cells were counted daily and cultures (with and without 1 µg/ml tetracycline) were washed three times and induced for expression of the ectopic epimerase under the control of the tetracycline repressor (TETR) protein. Northern blot analysis showed that TbGALE mRNA was more abundant in the conditional mutant than in wild type cells (Fig. 5C, compare lanes 1 and 2), but that it became undetectable within 6 h of tetracycline removal (Fig. 5C, lane 5). Northern blot analysis of the RNA of cells that spontaneously grew after 15 days showed that they had undergone genetic rearrangement to escape tetracycline-control and to constitutively expressed the ectopic TbGALE gene (Fig. 5C, lane 7). The inclusion of 10 mM Gal in the medium did not alter the results (data not shown).

T. brucei UDP-Glc 4’-epimerase Single-Allele Knockout Clones Exhibit Haploid-Insufficiency with Respect to Procyclin Galactosylation - We analysed whole cell lysates from wild-type cells and from two independent single-allele knockout clones (ΔTbGALE::PAC) and (ΔTbGALE::BSR) by SDS-PAGE and Western blotting with anti-EP-procyclin antibodies. This revealed a reduction in procyclin apparent molecular weight in the single-allele knockout cells (Fig. 6A). We also extracted procyclins from the same cells and analysed them by negative-ion MALDI-ToF following aq. HF dephosphorylation and mild-acid treatment (Fig. 6B). This showed that only EP1-1, EP1-2 and EP3 procyclins were being expressed.

Although the higher proportion of the shorter EP3 procyclin in ΔTbGALE::BSR cells would account for a slight decrease in procyclin average molecular weight (about 0.3 kDa), it seemed likely that the majority of the observed reduction in procyclin apparent molecular weight would be due to changes in the GPI anchor side-chains, the only site of galactosylation in procyclins (6, 20). Therefore, we measured the Man and Gal content of the procyclins by GC-MS following methanolsysis and TMS-derivatisation (33) and normalized the figures to Man = 7.0 (Table I). EP1-1, EP1-2 and EP3 Procyclins contain 7 measurable Man residues per molecule because all three contain the same single Man3GlcNAc2 N-linked glycan (6, 12, 37) and because only 2 out of the 3 Man residues of their GPI anchors can be liberated as free mannose by methanolsysis (38). The data suggest that wild type procyclins contain an average of 11.8±1.4 Gal residues whereas the single-allele UDP-Glc 4’-epimerase knockout mutants contain an average of 8.4±0.6 Gal residues (Table I). This 30% reduction in Gal content suggested that procyclic form T. brucei suffer from haploid insufficiency with respect to procyclin galactosylation but that this insufficiency has negligible effects on growth rate in vitro.

Procyclic Form T. brucei TbGALE Conditional Null Mutant Cells Express a High Copy
Number of Hypo-Galactosylated Procyclin Molecules Under Non-Permissive Conditions - Procyclin samples were extracted from TbGALE conditional null mutant cells 0, 5 and 9 days following the removal of tetracycline from the medium. SDS-PAGE and Western blot analysis with anti-EP procyclin antibodies revealed that the day-5 and day-9 procyclins had significantly lower apparent molecular weights than the day-0 material (data not shown). The samples were re-analysed following mild acid treatment, a procedure that cleaves EP-procyclins at the Asp-Pro bonds present before the (EP)_n-GPI domain in all EP-procyclins and that simplifies the SDS-PAGE pattern of these molecules (12). A similar reduction in apparent molecular weight was observed (Fig. 7A), suggesting that the molecular weight shift was due to changes in the C-terminal portion of the molecule. Negative ion MALDI-Tof analysis of the aq.HF dephosphorylated/mild-acid treated procyclins from day-0 and day-9 showed that only EP1-1, EP1-2 and EP3 procyclins were present and that the relative proportions of these isoforms were very similar (Fig. 7B). An analysis of aq.HF dephosphorylated material without mild acid treatment revealed the presence of the same Man5GlcNAc2 N-linked oligosaccharide on all procyclin species (data not shown). The mass spectrometric data, therefore, confirmed that changes in apparent molecular weight observed in (Fig. 7A) were indeed due to changes in the C-terminal portion of the EP procyclins.

The anti-EP procyclin Western blot also suggested a substantial increase in the amount of (lower-molecular weight) EP procyclin at day-9 (compare Fig. 7A, lane 1 with lane 3). To analyse this directly, we measured the absolute molar quantity of procyclins in the day-0 and day-9 samples using a GC-MS method that quantifies the GPI component of a sample by measuring the non-N-acetylated glucosamine content (32). The results showed that procyclin expression was approximately 10-fold higher in the day-9 cells compared to the day-0 cells (Table I). We further analysed the Man: Gal ratio in these samples by GC-MS and found that the day-9 procyclins were hypo-galactosylated and contained, on average, 27.5-fold less Gal than day-0 procyclins; i.e., an average of 0.3 Gal residues per molecule (Table I). Finally, we noted that cells that resumed growth after day-15, due to constitutive expression of the ectopic TbGALE gene, restored their expression of higher molecular weight procyclin (Fig. 7A, lane 4).

DISCUSSION

T. brucei UDP-Glc 4' -epimerase is a typical dimeric (Fig. 1) NADH-dependent oxidoreductase (EC 5.1.3.2) that interconverts UDP-Glc and UDP-Gal. However, unlike the human epimerase (39), the T. brucei enzyme is unable to inter-convert UDP-GlcNAc and UDP-GalNAc (Fig. 2). The same is true for the T. cruzi enzyme (35) and this almost certainly explains why GalNAc has not been found in any trypanosome glycoconjugates.

The epimerase appears to be located in glycosomes in the bloodstream and procyclic form of T. brucei according to immunofluorescence microscopy (Fig. 3A-F) and in bloodstream forms by subcellular fractionation and Western blotting (Fig. 3G). Subcellular fractionation and Western blotting was also attempted with procyclic cells but there was insufficient epimerase in these cells to allow Western blot detection. This low level of epimerase expression in procyclic cells but there was insufficient epimerase in these cells to allow Western blot detection. This low level of epimerase expression in procyclic cells suggests that GalNAc is not found in any trypanosome glycoconjugates.

The presence of UDP-Glc 4’-epimerase in the glycosome suggests a complex compartmentalization of sugar nucleotide biosynthesis such that the product of hexokinase and phosphoglucose mutase, glucose-1-phosphate, is presumably transported out of the glycosome and into the cytosol via UTP:glucose-1-phosphate uridylyltransferase to form UDP-Glc. The putative T. brucei UTP:glucose-1-phosphate uridylyltransferase gene sequence (Tb10.389.0330) predicts neither a PTS1- nor PTS2-type glycosomal import signal and is presumed to be cytoplasmic. Thus, UDP-Glc made in the cytoplasm most likely enters the glycosome, via a specific sugar nucleotide transporter or pore, to be epimerised into UDP-Gal. The UDP-Gal must then be transported back to the
cytoplasm and, from there, into the lumen of the ER and the Golgi apparatus for use by a range of UDP-Gal-dependent α- and β-Gal transferases. Why the UDP-Glc/UDP-Gal epimerization process would need to be compartmentalized in this way is not clear.

The UDP-Glc 4’-epimerase, and therefore galactose metabolism, has been shown to be essential in bloodstream form *T. brucei* (24). Similarly, we were unable to create a *TbGALE* null mutant in procyclic form *T. brucei* and the cessation of growth under non-permissive conditions in a procyclic form conditional null mutant (Fig. 5) supports the conclusion that UDP-Glc 4’-epimerase activity is also essential for cell growth in this life-cycle stage of the parasite. The long delay (7-8 days) between the suppression of *TbGALE* mRNA transcription and the cessation of cell growth could be due to high *TbGALE* mRNA levels in the tetracycline-induced conditional null mutant, leading to increased epimerase protein levels, combined with a relatively long protein half-life. Thus, it could take several cell-divisions to reduce epimerase levels to unsustainable levels. The inability of free Gal in the medium to rescue the conditional null mutants shows that, like bloodstream form *T. brucei*, procyclic form *T. brucei* is unable to take up (43) and metabolize Gal by the Leloir pathway (44).

The sugar nucleotide UDP-Gal is the ubiquitous donor for eukaryote galactosyltransferases. Therefore, the most likely explanation for the essentiality of the *TbGALE* gene is an absolute requirement for one or more Gal-containing oligosaccharides on one or more parasite glycoproteins. In bloodstream form parasites, there are several known Gal-containing glycoproteins. All VSG variants contain Gal in their N-linked oligosaccharides and/or GPI membrane anchor side-chains (38, 45-47) and, according to ricin binding (48), tomato lectin binding (49) and structural characterization (50), several other glycoproteins in the flagellar pocket and endosomal/lysosomal system contain poly-N-acetyl-lactosamine glycans. Some of these N-linked poly-N-acetyl-lactosamine glycans are extremely large and of unusual structure (50). In procyclic form *T. brucei*, these large poly-N-acetyl-lactosamine glycans are absent and the N-linked oligosaccharide repertoire is limited to oligomannose (Manα1,6GlcNAc2) structures (6, 47, 51, 52). Thus, in this life-cycle stage, Gal appears to be largely restricted to the poly-N-acetyl-lactosamine-containing side-chains of the procyclin GPI anchors (6, 47) and of the free GIPLs (7-9). An obvious hypothesis is that these GPI side-chain poly-N-acetyl-lactosamine structures may play an important (possibly anti-adhesive) protective role on the cell surface. A way to test this would be to make null, conditional null or inducible RNAi mutants for the βGal- and βGlcNAc-transferases involved in poly-N-acetyl-lactosamine synthesis. However, this is not yet experimentally amenable because bioinformatic analysis has not provided obvious candidates for these genes.

Thus far, the normal molecular architecture of procyclic form *T. brucei* has been perturbed by gene knockouts of procyclin genes and by gene knockouts and RNAi of GPI biosynthesis genes. The *TbGPI10* and *TbGP18* genes, that encode the third α-mannosyltransferase and the catalytic subunit of the GPI:protein transamidase, respectively, are essential for bloodstream form *T. brucei* but non-essential for the procyclic form in culture, provided non-adherent tissue culture ware is used (8, 9). GPI-minus *TbGPI10* and *TbGPI18* knockout cells do not express procyclin on their surface and are significantly impaired (particularly the *TbGPI18* knockout) in their ability to colonize the tsetse fly midgut. Analysis of these cells and total procyclin gene (*GPEET, EP1, EP2* and *EP3*) knockout cells (7) revealed that the absence of cell-surface procyclin is compensated for by an increase in the copy number of GIPLs that are, in essence, GPI anchors not attached to protein and with fewer (about 4) N-acetyl-lactosamine repeats (Acosta-Serrano and Ferguson, unpublished data). In this paper, we have analyzed the results of a different kind of cell surface perturbation, brought about by galactose-starvation using procyclic form *T. brucei* *TbGALE* single allele knockout and *TbGALE* conditional null mutant cells.

The *TbGALE* single allele knockout clones exhibited a reduction in the apparent molecular weights of their EP procyclins (Fig. 6) that was traced to a reduction in their Gal-content of about 30% (Table I). This clearly had little or no impact on cell viability but serves to demonstrate that procyclic form *T. brucei* does not express an excess of epimerase activity to supply its galactosylation needs. Indeed, the barely detectable levels of *TbGALE* mRNA and undetectable levels of epimerase protein in wild-type procyclic cells is consistent with this notion. A similar example of haploid insufficiency with respect
to a biochemical phenotype (rather than viability) was reported for the ConA 1-1 procyclic form mutant that lacks one functional allele of polyprenol reductase, an enzyme involved in synthesis of dolichol. This partial defect affected procyclin N-glycosylation and rendered the parasites resistant to killing by the lectin Concanavalin A (52).

A more dramatic phenotype than that of the TbGALE single-allele knockout was observed with the epimerase conditional null mutant following the withdrawal of tetracycline (Fig. 7A). In this case, after 9 days, when growth essentially ceased, the cells still expressed the same EP procyclins as the tetracycline-induced control cells but with dramatically lower apparent molecular weights. The entire reduction in molecular weight was mapped to the GPI anchor side-chains that were almost entirely free of galactose (Table I). This reduction in size correlated with a 10-fold upregulation in procyclin protein expression, presumably an attempt to compensate for the parasite’s inability to cover the cell surface with bulky trans-sialylated poly-N-acetyl-lactosamine units. Thus, while procyclic form T. brucei appears to be able to compensate for a lack of procyclin protein by upregulation of poly-N-acetyl-lactosamine-containing GIPLs (7-9) the converse is not the case (Fig. 8). We suggest, therefore, that the primary role of procyclins (at least in the early stages of parasite differentiation and tsetse fly colonization) is to act as a platform for the efficient expression of anti-adhesive, membrane-protecting trans-sialylated poly-N-acetyl-lactosamine oligosaccharides.

REFERENCES

1. Cross, G. A. (1996) *Bioessays* **18**, 283-291
2. Vanhamme, L., Pays, E., McCulloch, R., and Barry, J. D. (2001) *Parasitol. Today* **17**, 338-343
3. Roditi, I., Carrington, M., and Turner, M. (1987) *Nature* **325**, 272-274
4. Mowatt, M. R., and Clayton, C. E. (1987) *Mol. Cell. Biol.* **7**, 2838-2844
5. Richardson, J. P., Beecroft, R. P., Tolson, D. L., Liu, M. K., and Pearson, T. W. (1988) *Mol. Biochem. Parasitol.* **31**, 203-216
6. Treumann, A., Zitzmann, N., Hülsmeier, A., Prescott, A. R., Almond, A., Sheehan, J., and Ferguson, M. A. J. (1997) *J. Mol. Biol.* **269**, 529-547
7. Vassella, E., Butikofer, P., Engstler, M., Jelk, J., and Roditi, I. (2003) *Mol. Biol. Cell* **14**, 1308-1318
8. Lillico, S., Field, M. C., Blundell, P., Coombs, G. H., and Mottram, J. C. (2003) *Mol. Biol. Cell* **14**, 1182-1194
9. Nagamune, K., Acosta-Serrano, A., Uemura, H., Brun, R., Kunz-Renggli, C., Maeda, Y., Ferguson, M. A., and Kinoshita, T. (2004) *J. Exp. Med.* **199**, 1445-1450
10. Roditi, I., Schwarz, H., Pearson, T. W., Beecroft, R. P., Liu, M. K., Richardson, J. P., Bühning, H.-J., Pleiss, J., Bülow, R., Williams, R. O., and Overath, P. (1989) *J. Cell. Biol.* **108**, 737-746
11. Roditi, I., and Clayton, C. (1999) *Mol. Biochem. Parasitol.* **103**, 99-100
12. Acosta-Serrano, A., Cole, R. N., Mehlert, A., Lee, M. G., Ferguson, M. A., and Englund, P. T. (1999) *J. Biol. Chem.* **274**, 29763-29771
13. Mehlert, A., Treumann, A., and Ferguson, M. A. (1999) *Mol. Biochem. Parasitol.* **98**, 291-296
14. Butikofer, P., Vassella, E., Ruepp, S., Boschung, M., Civenni, G., Seebeck, T., Hemphill, A., Mookherjee, N., Pearson, T. W., and Roditi, I. (1999) *J. Cell. Sci.* **112**, 1785-1795
15. Schlaeppi, A. C., Malherbe, T., and Butikofer, P. (2003) *J. Biol. Chem.* **278**, 49980-49987
16. Vassella, E., Den Abbeele, J. V., Butikofer, P., Renggli, C. K., Furger, A., Brun, R., and Roditi, I. (2000) *Genes Dev.* **14**, 615-626
17. Morris, J. C., Wang, Z., Drew, M. E., and Englund, P. T. (2002) *EMBO J.* **21**, 4429-4438
18. Vassella, E., Probst, M., Schneider, A., Studer, E., Renggli, C. K., and Roditi, I. (2004) *Mol. Biol. Cell* **15**, 3986-3993
19. Acosta-Serrano, A., Vassella, E., Liniger, M., Kunz Renggli, C., Brun, R., Roditi, I., and Englund, P. T. (2001) *Proc. Natl. Acad. Sci. U S A* **98**, 1513-1518
20. Ferguson, M. A. J., Murray, P., Rutherford, H., and McConville, M. J. (1993) *Biochem. J.* **291**, 51-55
21. Engstler, M., Reuter, G., and Schauer, R. (1993) *Mol. Biochem. Parasitol.* **61**, 1-14
22. Pontes de Carvalho, L. C., S., T., F., Bienen, E. J., Clarkson, A. B., Jiang, M. S., Hart, G. W., and Nussenzweig, V. (1993) J. Exp. Med. 177, 465-474
23. Montagna, G., Cremona, M. L., Paris, G., Amaya, M. F., Buschiazzo, A., Alzari, P. M., and Frasch, A. C. (2002) Eur. J. Biochem. 269, 2941-2950
24. Roper, J. R., Guthier, M. L., Milne, K. G., and Ferguson, M. A. (2002) Proc. Natl. Acad. Sci. U S A 99, 5884-5889
25. Brun, R., and Schönenberger, M. (1979) Acta Tropica 36, 289-292
26. Hirumi, H., and Hirumi, K. (1989) J. Parasitol. 75, 985-989
27. Schuck, P. (2004) Biophys. Chem. 108, 187-200
28. Schneider, P., McConville, M. J., and Ferguson, M. A. J. (1994) J. Biol. Chem. 269, 18332-18337
29. Cross, G. A. (1984) J. Parasitol. 24, 79-90
30. Steiger, R.F., Opperdoes, F.R., and Bontemps, J. (1980) Eur. J. Biochem. 105, 163-175
31. van den Hoff, M.J., Moorman, A.F., and Lamers, W.H. (1992) Nucleic Acids Res. 20, 2902-2906
32. Roper, J. R., and Ferguson, M. A. J. (1999) Glycobiology: A Practical Approach. Fukuda, M. and Kobata, A. (eds.) IRL Oxford Univ. Press. pp349-383.
33. Shaw, M. P., Bond, C. S., Roper, J. R., Gourley, D. G., Ferguson, M. A., and Hunter, W. N. (2003) Mol. Biochem. Parasitol. 126, 173-180
34. Roper, J. R., and Ferguson, M. A. (2003) Mol. Biochem. Parasitol. 132, 47-53
35. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) Mol. Biochem. Parasitol. 99, 89-101
36. Michels, P. A., Hannaert, V., and Bringaud, F. (2000) Parasitol. Today 16, 482-489
37. Parsons, M. (2004) Mol. Microbiol. 53, 717-724
38. Acosta-Serrano, A., Cole, R. N., and Englund, P. T. (2000) J. Mol. Biol. 304, 633-644
39. Ferguson, M. A. J., Homans, S. W., Dwek, R. A., and Rademacher, T. W. (1988) Science 239, 753-759
40. Thoden, J. B., Wohlers, T. M., Fridovich-Keil, J. L., and Holden, H. M. (2001) J. Biol. Chem. 276, 15131-15136
41. Nolan, D. P., Geuskens, M., and Pays, E. (1999) Current Biology 9, 1169-1172
42. Attri, A., Richardson, J. M., Prescott, A. R., and Ferguson, M. A. J. (2005) J. Biol. Chem. 280, 865-871
43. Hwa, K. Y., and Khoo, K. H. (2000) Mol. Biochem. Parasitol. 111, 173-184
44. Acosta-Serrano, A., O’Rear, J., Quellhorst, G., Lee, S. H., Hwa, K. Y., Krag, S. S., and Englund, P. T. (2004) Eukaryot. Cell 3, 255-263

FOOTNOTES

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The abbreviations used are: VSG, variant surface glycoprotein; GIPLs, glycoinositol phospholipids; GPI, glycosylphosphatidylinositol; PAC, puromycin acetyl transferase; BSR, blasticidin resistance; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde phosphate dehydrogenase; TFA, trifluoroacetic acid.

FIGURE LEGENDS

Fig. 1. Estimation of the molecular weight of recombinant *T. brucei* UDP-Glc 4′-epimerase by analytical ultracentrifugation. SEDFIT analysis of sedimentation velocity data from recombinant *T. brucei* enzyme in PBS at 1 mg/ml.

Fig. 2. HPLC assay of sugar nucleotide inter-conversion by recombinant *T. brucei* UDP-Glc 4′-epimerase. The *T. brucei* epimerase was incubated with UDP-Glc (panel A), UDP-Gal (panel B), UDP-GlcNAc (panel C) or UDP-GalNAc (panel D) and the products were resolved by SAX-PLC in borate buffer and detected using a UV-monitor at 262 nm. The elution positions of authentic UDP-sugar standards are indicated above the chromatograms. The small peaks at 19 and 32 min are UMP and UDP, respectively, from partial decomposition of the UDP-sugar substrates.

Fig. 3. Subcellular localisation of *T. brucei* UDP-Glc 4′-epimerase in bloodstream form and procyclic form *T. brucei*. Merged phase-contrast and FITC-fluorescence microscopy images of fixed bloodstream form (panels A, C and E) and procyclic form (panels B, D and F) trypanosomes. The cells were stained for immunofluorescence with rabbit polyclonal antibodies to recombinant *T. brucei* UDP-Glc 4′-epimerase (panels A and B) or to the *T. brucei* glycosome marker GAPDH (panels C and D). Control incubations with pre-immune sera are also shown (panels E and F). Panel G: Western blot of subcellular fractions of bloodstream form trypanosomes (equivalent to 2.9 x 10⁸ cells per lane) using rabbit polyclonal antibodies to recombinant *T. brucei* UDP-Glc 4′-epimerase. The subcellular fractions are large granular (LG), small granular (SG), microsomal (M) and cytosolic (C) (lanes 1-4, respectively). A positive control of His₆-tagged recombinant epimerase (rec) is shown in (lane 5).

Fig. 4. Southern blot characterisation of procyclic single-allele knockout and conditional null mutants. Southern blots of genomic DNA digested with *Pst* I were probed with *TbGALE* ORF (*GALE*), the 5′-UTR of *TbGALE* (5′-UTR), the blasticidin resistance selectable marker gene (*BSR*) or the puromycin resistance selectable marker gene (*PAC*). Lane 1, wild type cells; lane 2, *ΔTbGALE::BSR*; lane 3, *ΔTbGALE::PAC*; lane 4, *ΔTbGALE::ΔTbGALE::PAC* (clone 1); lane 5, *ΔTbGALE::ΔTbGALE::PAC* (clone 2); lanes 6-9, *ΔTbGALE::ΔTbGALE::PAC* (clones 1-4). Note: *ΔTbGALE::ΔTbGALE::PAC* clone 2 (lane 5) was used to create all four conditional null mutant clones (lanes 5-9).

Fig. 5. *TbGALE* is essential for the growth of procyclic form *T. brucei*. Panel A: Continuous growth of a *ΔTbGALE::ΔTbGALE::BSR/ΔTbGALE::PAC* conditional null mutant in the presence of tetracycline. Panel B: Continuous growth followed by retarded growth and, eventually, recovery of the same clone in the absence of tetracycline. Panel C: Northern blot of RNA isolated from wild type cells (lane 1), the mutant cells 0, 2, 4, 6, 24 h after removal of tetracycline (lanes 2-6, respectively) and the mutant cells after growth had resumed after 15 days without tetracycline (lane 7).

Fig. 6. Gene replacement of one allele of *TbGALE* results in the expression of smaller procyclin molecules. Panel A: Anti-procyclin Western blot of cell lysates from wild type and two independent single allele knockout clones of procyclic form *T. brucei*. Molecular weight markers are shown on the left. Panel B: MALDI-Tof mass spectra of aq. HF dephosphorylated and mild acid-treated procyclins from wild type (top), *ΔTbGALE::BSR* single allele knockout cells (middle) and *ΔTbGALE::PAC* single allele knockout cells (bottom). The diagnostic peptide ions (12) for EP1-1, EP1-2 and EP3 procyclins are indicated.
Fig. 7. Galactose starvation causes an upregulation in the expression of hypo-galactosylated procyclin. Panel A: SDS-PAGE and anti-procyclin Western blot of mild-acid treated procyclins from the UDP-Glc 4’-epimerase conditional (tetracycline-inducible) null mutant 0, 5 and 9 days after withdrawal of tetracycline (lanes 1-3) and following the resumption of cell growth after 15 days (lane 4). Panel B: MALDI-Tof mass spectra of aq. HF dephosphorylated and mild acid-treated procyclins from the UDP-Glc 4’-epimerase conditional (tetracycline-inducible) null mutant 0 and 9 days after withdrawal of tetracycline. The diagnostic peptide ions (12) for EP1-1, EP1-2 and EP3 procyclins are indicated.

Fig. 8. Comparison of procyclic form T.brucei mutant phenotypes. Wild-type procyclic form T.brucei cells express a total of about 4 x 10^6 GPI molecules (32) of which about 3 x 10^6 (6) are attached to procyclins and the balance are presumably mostly cell-surface GIPLs (7-9). The procyclins have GPI side-chains of about 10 N-acetyl-lactosamine (Galβ1-4GlcNAc) units with about 5 terminal sialic acid residues (6, 20) while the GIPL side-chains are substantially smaller with about 4 N-acetyl-lactosamine units (Acosta Serrano and Ferguson, unpublished data). Deletion of all procyclin genes (7) or RNAi of TbGPI8 (8) or gene deletion of TbGPI10 or TbGPI8 (9) leads to the upregulation of GIPL expression and cell survival in culture. On the other hand, galactose starvation in TbGALE conditional null mutants under non-permissive conditions (this study) leads to 10-fold upregulation of procyclin protein expression but the cells stop growing once GPI N-acetyl-lactosamine side-chains are depleted.
### TABLE I

*Procyclin compositional data.*

| Procyclin sample | Copy number per cell | (GlcN content)<sup>a</sup> | Man<sup>b</sup> | Gal<sup>b</sup> |
|------------------|----------------------|--------------------------|----------------|-----------------|
| Wild-type *TbGALE*<sup>+/+</sup> cells | nd | 7.0 | 11.8 ± 1.4 |
| Δ*TbGALE::PAC* single-allele KO | nd | 7.0 | 8.7 ± 0.5 |
| Δ*TbGALE::BSR* single-allele KO | nd | 7.0 | 8.4 ± 0.6 |
| *TbGALE* conditional null cells, day 0 | 2.8 ± 0.6 x 10<sup>6</sup> | 7.0 | 7.8 ± 1.9 |
| *TbGALE* conditional null cells, day 9 | 2.6 ± 0.2 x 10<sup>7</sup> | 7.0 | 0.3 ± 0.05 |

**Notes:**

All figures are the means of triplicate analyses ± 1 standard deviation.

nd = not determined.

<sup>a</sup>Quantified by GC-MS according to (32).

<sup>b</sup>Quantified by GC-MS according to (33). Ratio fixed to Man = 7.0 to reflect the 7 Man residues per mol procyclin (5 from the Man<sub>3</sub>GlcNAc<sub>2</sub> N-linked oligosaccharide and 2 from the GPI anchor) that are detected by this method.
A

| kDa | MW | Wild-type | ΔGALE::BSR | ΔGALE::PAC |
|-----|----|-----------|------------|------------|
| 52  |    |           |            |            |
| 31  |    |           |            |            |
| 19  |    |           |            |            |
| 17  |    |           |            |            |
| 11  |    |           |            |            |
| 6   |    |           |            |            |
| 3   |    |           |            |            |

Procyclin

B

Wild-type

EP1-2

EP1-1

ΔGALE::BSR

ΔGALE::PURO
Elimination of procyclin expression (refs. 7-9)

Galactose starvation (this study)

Upregulation of GIPL expression and survival in culture

Upregulation of procyclin expression but non-survival in culture
The suppression of galactose metabolism in procyclic form trypanosoma Brucei causes cessation of cell growth and alters procyclin glycoprotein structure and copy number
Janine R. Roper, M. Lucia S. Guth, James I. MacRae, Alan R. Prescott, Irene Hallyburton, Alvaro Acosta-Serrano and Micheal A. J. Ferguson

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