THE DE NOVO SYNTHESIS OF GDP-FUCOSE IS ESSENTIAL FOR FLAGELLAR ADHESION AND CELL GROWTH IN TRYPANOSOMA BRUCEI*

Daniel C. Turnock, Luis Izquierdo and Michael A.J. Ferguson

Division of Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee

Running head – The synthesis and role of GDP-fucose in T. brucei

Address correspondence to Michael A.J. Ferguson, MSI/WTB complex, Dow Street, Dundee, DD15EH, UK. Telephone -44-1382-384219 Fax: -44-1382-348896
Email: m.a.j.ferguson@dundee.ac.uk

The protozoan parasite Trypanosoma brucei causes human African sleeping sickness in sub-Saharan Africa. The parasite makes several essential glycoproteins, which has led to the investigation of the sugar nucleotides and glycosyltransferases required to synthesize these structures. Fucose is a common sugar in glycoconjugates from many organisms, however, the sugar nucleotide donor GDP-fucose was only recently detected in T. brucei and the importance of fucose metabolism in this organism is not known. In this paper, we identified the genes encoding functional GDP-fucose biosynthesis enzymes in T. brucei and created conditional null mutants of TbGMD, the gene encoding the first enzyme in the pathway from GDP-mannose to GDP-fucose, in both bloodstream form and procyclic form parasites. Under non-permissive conditions, both lifecycle forms of the parasite became depleted in GDP-fucose and suffered growth arrest, demonstrating that fucose metabolism is essential to both lifecycle stages. In procyclic form parasites, flagellar detachment from the cell body was also observed under non-permissive conditions, suggesting that fucose plays a significant role in flagellar adhesion. Fluorescence microscopy of epitope-tagged TbGMD revealed that this enzyme is localised in glycosomes, despite the absence of PTS-1 or PTS-2 target sequences.

Trypanosomatid parasites are protozoan parasites that impose a major health burden on many countries in the developing world, causing a wide range of diseases over three continents. Trypanosoma brucei is the causative agent of African sleeping sickness in humans and nagana in cattle and is spread between mammalian hosts by the bite of the tsetse fly (Glossina spp.). T. brucei transmission occurs when the bloodstream form of the parasite is ingested by a tsetse fly during feeding, the parasite then differentiates into the procyclic form in order to colonise the tsetse fly mid gut. The parasite undergoes further differentiation and migration to the salivary gland of the fly in order to infect a new mammalian host upon a subsequent bloodmeal. Research in several laboratories has focused on the glycobiology of these organisms to uncover potential targets for therapeutic intervention (1-5). These targets include enzymes of glycosylphosphatidylinositol (GPI) biosynthesis (1,6-9) and enzymes of sugar nucleotide biosynthesis. For example, GDP-mannose biosynthesis has been shown to be essential for the infectivity of Leishmania mexicana (10-13) and UDP-glucose 4’-epimerase (GalE), the only source of UDP-galactose in T. brucei, has been shown to be essential for both bloodstream form and procyclic form T. brucei (14-16) and is likely to be essential for epimastigote form T. cruzi (17).

Sugar nucleotides are activated forms of sugars that are used as the ultimate source of sugar for the majority of glycosylation reactions. Sugar nucleotides are formed in two main ways; by a salvage pathway involving “activation” of the sugar using a kinase and a pyrophosphorylase or by a “de
"de novo" pathway involving the bioconversion of an existing sugar/sugar nucleotide. In most cases, sugar nucleotides are synthesized in the cytoplasm and then transported through specific transporters into the lumen of the Golgi apparatus and/or endoplasmic reticulum (ER) where they are used by glycosyltransferases as donor substrates in glycosylation reactions (18,19).

The sugar nucleotide GDP–fucose has been found in a wide variety of different organisms, ranging from bacteria to humans (20,21). Two pathways for the biosynthesis of GDP-fucose have been identified. The first is a three step conversion of GDP-mannose to GDP-fucose catalysed by two enzymes, a GDP-mannose dehydratase (GMD; EC 4.2.1.47) and a GDP–fucose synthetase, also known as GDP-4-dehydro-6-deoxy-D-mannose epimerase/reductase (GMER; EC 1.1.1.271) (20). The second is a salvage pathway involving the phosphorylation of fucose by a fucose kinase, followed by condensation with GTP catalysed by a fucose-1-phosphate pyrophosphorylase (20). The enzymes involved in these conversions have been isolated and/or characterised from a variety of sources including bacteria (22), mammals (23-26) and plants (27). Homologues of the “de novo” GDP-fucose biosynthesis enzymes were identified in the recent Trypanosomatid genome projects (28-30).

Relatively little is known about the role of fucose in the trypanosomatid parasites. In T. cruzi, fucose has been found as a minor component in the N-glycans of the cruzipain cysteine protease (31) and in a glycoprotein called gp72, where it is found together with rhamnose, xylose, galactopyranose, galactofuranose and N-acetylglucosamine in unique complex glycans attached to Thr and Ser via phosphodiester linkages (32-35). Gp72 is non-essential in culture but has a role in flagellar adhesion in the epimastigote form of the parasite. Thus, the epimastigote form of T. cruzi has a detached flagellum when the gp72 gene is deleted (36), even though the rest of the flagellar adhesion zone (FAZ) structure appears to be normal, as judged by electron and light microscopy (37). Other experiments have shown that gp72 is required for infection of both the insect vector and mammalian host (38,39). However, the exact role of the fucose containing glycan in the function of the protein is not known. No fucosylated structures have been identified in T. brucei to date. However, T. brucei has two homologues of gp72, called Fla-1 (40) and Fla-2 (41). Inducible RNAi studies have shown that knockdown of Fla-1 mRNA in T. brucei, like the gp72 null mutant in T. cruzi, caused a defect in flagellar adhesion (41). This suggests, indirectly, that Fla-1, and possibly Fla-2, is/are candidates for being fucosylated glycoproteins in T. brucei.

In this work, we demonstrate that the putative T. brucei GDP-fucose biosynthesis enzymes are functional in vitro by expressing them as glutathione S-transferase (GST) fusion proteins in E. coli and then detecting GDP-fucose production using a liquid chromatography – tandem mass spectrometry (LC-MS/MS) based assay. We analysed the function of fucose in T. brucei by making a conditional null mutant in the first enzyme of the GDP-fucose biosynthesis pathway in both the bloodstream and procyclic form lifecycle stages. We show that mutation of the T. brucei GDP-mannose 4,6-dehydratase gene (TbgMD) results in loss of GDP-fucose from intracellular pools, a reduction in growth rate and flagellar detachment in the procyclic form. We also show that epitope tagged TbGMD protein localises to the glycosome in procyclic form T. brucei.

Experimental Procedures

Parasite culture - T. brucei bloodstream form parasites (strain 427, variant MITaT1.2) that express T7 polymerase and tetracycline repressor protein under G418 selection (42) were cultured in HMI-9
medium (43) up to a maximum density of 3 x 10^6 cells/ml at 37°C with 5% CO_2. For extraction of genomic DNA, RNA and sugar nucleotides, cells were harvested at ~1 x 10^6/ml. T. brucei procyclic form parasites that express T7 polymerase and tetracycline repressor protein under G418 and hygromycin selection (cell line 29:13) were cultured in SDM-79 medium (44) up to a maximum density of 5 x 10^7 cells/ml at 28°C. For extraction of genomic DNA, RNA and sugar nucleotides, cells were harvested at ~1 x 10^7/ml.

**Cloning, expression and assay of GDP-fucose biosynthesis genes in E.coli -** For over-expression in E. coli, the TbGMD and TbGMER genes were amplified by PCR with the following forward and reverse primers to add either Bam HI, Eco RI or Xho I restriction sites (underlined):

5’ cccgggatcc ATGTCAGCACGTCGACTGGC 3’

5’ gcgaattc CTATTGCCCCGCTGCTCAAC AC 3’ for TbGMD, and

5’ cccgggatcc ATGTTAGGGTCCCTACCGAGTT 3’

5’ cgccctcgag CTTCCGTGCGACATCGTAGTT 3’ for TbGMER

The PCR products were digested with appropriate restriction enzymes and cloned into the pGEX 4T expression vector (Amersham) and expressed in BL21 (DE3) E. coli cells. Cultures of 1L culture were grown to an OD_600 of 0.5 and expression was induced overnight at room temperature with 100 µM Isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested and lysed in phosphate buffered saline (PBS) with 1 mg/ml lysozyme for 30 min on ice. Complete lysis was ensured by sonication (soniprep-150, Sanyo) or cell disruption (One shot cell disruptor, Constant cell disruption systems) in the presence of Complete protease inhibitor cocktail (Roche). The lysate was clarified by centrifugation at 30,000 g for 20 min before adding the supernatant to ~300 µl of PBS-washed GST beads (Amersham) and incubating for 1 hour at 4°C. The beads were then washed with >10 bead volumes of PBS before 3 elutions of 300 µl with 10 mM glutathione in 50 mM Tris-HCl pH 7.4.

Assay conditions were adapted from (23). Reactions were performed in 100 µl of 100 mM 3-(n-Morpholino)propanesulfonic acid (MOPS) pH 7.0, 100 mM NaCl, 10 mM dithiothreitol (DTT), 5 mM Ethylenediaminetetraacetic acid (EDTA), 25 µM GDP-mannose (Sigma), 10 µM NADPH and 100 µM NADP. The reaction was started by adding ~25 µg of the recombinant TbGMD enzyme (pre-incubated at ~1mg/ml with 100 µM NADP for 30 mins at 4°C) and the reaction was left to proceed for 1 h at 37°C. Recombinant TbGMER (~25 µg) was then added and the concentration of NADPH was adjusted to 150 µM. For experiments without the TbGMER enzyme, only NADPH was added at this point. Reactions were stopped after 1h at 37°C by heating to 100°C for 2 minutes and then the samples were centrifuged at 15,000 rpm to remove insoluble material. Sugar nucleotides were purified using EnviCarb columns (45) and analysed by LC-MS/MS (46).

**Cloning and over expression of TbGMD in T. brucei -** The TbGMD ORF was amplified from genomic DNA by PCR using the forward and reverse primers to add either a Hind III or Pac I restriction site (underlined):

5’ cccagcctATGTCAGCACGTCGACTGGC 3’

5’ cccttaata GCTGGTTTCGGCGGTAATGC 3’, respectively.

The resulting PCR product was digested with Hind III and Pac I and cloned into a modified pLew82 vector (42) for expression with a C-terminal HA tag. The resulting construct was linearised with Not I and electroporated into procyclic cell line 29:13 (42). Transformants were selected with phleomycin and expression of the ectopic
copy was induced by addition of tetracycline at 0.5 µg/ml. The *TbGMD* ORF was also amplified from genomic DNA by PCR using primers

5'cccaagcttatgtcagcagctgactggc3' and

5'cccggttacgctgttgcggggtaatg3'.

The primers added either *Hind* III or *Bam* HI restriction sites (underlined) to the PCR products to allow cloning into the pLew100 expression vector (42) for use in constructing conditional null mutants.

**Immuno-fluorescence microscopy** - *T. brucei* procyclic form cells expressing HA tagged *TbGMD* were grown in SDM-79 to a cell density of ~1 x10^7/ml and approximately 2 x 10^7 cells were washed and re-suspended in PBS to a density of 4 x 10^7/ml. A small volume of cells (10-15 µl) were placed upon circular 13 mm coverslips (VWR) in a 12 well plate (Helena) and left at room temperature for 15 min to adhere. The cells were then fixed in 2 ml of 4% paraformaldehyde in PBS for 30 min and then washed three times in PBS. The coverslips were either stored at 4 °C in PBS plus 0.01% sodium azide or immediately prepared for immunofluorescence. Cells were permeabilised in 0.1% TX-100 in PBS for 20 min and then blocked for 1 hour in 0.5% BSA in PBS before staining with either rat anti-HA (1 in 20 dilution, Roche) or rabbit anti-GAPDH anti-serum (1 in 2000, a kind gift of Dr Opperdoes, Catholic University of Louvain) in 0.5% BSA, PBS. The coverslips were washed three times in PBS before staining with secondary antibody, either anti-rat FITC (1 in 30 dilution, Dako Cytomation) or anti-rabbit rhodamine (1 in 200 dilution, Dako Cytomation) as above. Excess secondary antibody was removed by washing three times in PBS. The coverslips were then mounted on polylysine slides using Hydromount (VWR) and left to dry in the dark for 30 min. Slides were examined on a Zeiss Axiovert 200 M fluorescence microscope and images were analysed and processed using Axiovision software.

**TbGMD gene replacements** - Approximately 500 bp of each of the *TbGMD* 5' and 3' UTR sequences were amplified from genomic DNA by PCR using primers:

5'ataagaattccgtggagacgtaacgactgtatgt3' and

5'gtttacttacagcagctgactggtcataatgaggtaatg3'.

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 *Hind* III and *Bam* HI cloning site (underlined in italics) and *Not* I restriction sites at each end (in bold). The PCR product was cloned into the pGEM-5Zf(+) vector (Promega) using the two *Not* I sites. To make the constructs for gene replacement in the procyclic form, the *PAC* and *BSD* drug resistance genes were simply introduced using the *Hind* III and *Bam* HI restriction sites. To make the constructs for bloodstream form electroporation, *HYG* and *PAC* genes flanked by a T7 promoter and two T7 terminator sequences were amplified by PCR using a modified pLew82 vector (42) containing either the *HYG* or *PAC* genes as a template. The primers used were 5'ccggaaccttaagataataacgactctacctaggg3' and 5'ccggaatctggggacctggccctcatttg3', which added Bst 98I and Bgl II restriction sites (underlined) respectively to the PCR products. The PCR products were digested with Bst 98I and Bgl II restriction enzymes and cloned into a Bst 98 I and *Bam* HI digested pGEM-5zF vector containing the 5' and 3' UTR flanking regions.
Transformation of 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 redissolved in sterile water. The linearized DNA was electroporated into either T. brucei bloodstream cells (strain 427, variant 221) or T. brucei procyclic form cells (strain 29:13) that were stably transformed to express T7 RNA polymerase and the tetracycline repressor protein under G418 selection (42). Cell culture and transformation was carried out as previously described (15,42).

Southern blotting - Genomic DNA was isolated from T. brucei cultures, digested with various restriction enzymes and separated by agarose gel electrophoresis. A fluorescein labelled TbGMD ORF probe was prepared by PCR using the primers 5’ TCTGCATTACGGAGATATGACG 3’ and 5’ACACCCAATCATCTGGCTTATC 3’. The probe was labelled using the Gene Images random prime labelling kit (GE Healthcare), 250 ng of template was used in a reaction volume of 50 µl and aliquots of 5µl were used per Southern Blot. The labelled probe was then detected using the Gene Images CDP-Star detection kit from (GE Healthcare).

RT-PCR - T. brucei RNA (1 µg) was extracted from 2 x 10^7 T. brucei cells using Qiagen midi RNeasy extraction kits with on-column DNAse digestion (RNAse free DNAse, Qiagen). RNA samples were then treated with Ominiscript reverse transcriptase (Qiagen) to generate cDNA. Both cDNA and RNA only control samples were then amplified by a standard PCR reaction using Taq polymerase and the TbGMD probe primers (see above). Alternatively, RT-PCR was carried out on approximately 50 ng of T. brucei RNA extracted from 5 x 10^6 cells using Qiagen mini RNeasy extraction kits with on-column DNAse digestion. Primers for RT-PCR were used at a final concentration of 1 µM (100 ng per reaction). RT-PCR was performed with an Access RT-PCR kit from Promega on a GeneAmp 2700 PCR cycler (Applied Biosystems).

Sugar nucleotide analysis - Sugar nucleotide analysis was performed as described elsewhere (46). Briefly, cells were pelleted by centrifugation, washed in ice-cold PBS, lysed in 70% ethanol in the presence of 10 pmol GDP-glucose internal standard and sugar nucleotides were extracted using Envi-Carb columns (45). Sugar nucleotides were then analysed using LC-MS/MS, using multiple reaction monitoring for detection. HPLC conditions were adapted from (45) and acetonitrile was added post-column to produce stable electrospray ionisation. The peak areas for each sugar nucleotide, along with their empirically determined molar relative response factors and the known amount of GDP-glucose internal, were used to quantify sugar nucleotides.

Scanning electron microscopy of T. brucei - Samples for scanning electron microscopy were prepared by the Centre for High Resolution Imaging and Processing (CHIPS), University of Dundee. Trypanosomes were fixed in either HMI-9 or SDM-79 media using 2.5% glutaraldehyde. Cells were collected on 1 µM Shandon nuclepore membrane filters and then rinsed twice for 15 min in 0.1 M sodium cacodylate buffer pH 7.2. Samples were treated with 0.2% osmium tetroxide overnight, and then washed twice for 15 min in water before washing in a gradient of ethanol solutions to dehydrate them. The samples were subjected to critical point drying using a BAL-Tec CPD D30, and mounted on aluminium stubs using carbon adhesive tabs. Finally, the stubs were coated with 40 nM Au/Pd using a Cressington 208HR sputter coater. The stubs were examined in a Philips XL30 ESEM operating at an accelerating voltage of 15 kV.

RESULTS
T. brucei has homologues of GDP-fucose biosynthesis genes - BLASTp searches (47) were performed against the T. brucei strain 927 predicted proteins database at GeneDB, using the amino acid sequences of the two human enzymes (GMD and GMER) involved in the de novo GDP-fucose biosynthesis from GDP-mannose as query sequences (accession numbers O60547 and Q13630). The putative T. brucei GMD gene returned was Tb10.61.0880 (accession number XP_828020). The same gene, referred to hereon as TbGMD, and its 5'- and 3'-UTR regions, were cloned from T. brucei strain 427 (accession number AM746334) and were virtually identical to the strain 927 sequences. The predicted protein product, TbGMD, has 49% sequence identity and 65% similarity to its human counterpart. The putative catalytic residues identified in the E. coli GMD crystal structure (48) can be found in the T. brucei sequence.

The GMER search returned a putative TbGMER gene (Tb11.01.5560, accession number XP_829455). This gene and its 5'- and 3'-UTR regions were cloned from T. brucei strain 427 (accession number AM746335) and were virtually identical to the strain 927 sequences with 56% identity and 76% similarity to its human counterpart. Homologues of GDP-fucose biosynthesis enzymes were also identified in T. cruzi (Tc00.1047053510027.10 and Tc00.1047053511727.300), which are both well conserved with their respective human and T. brucei counterparts. However, no homologues of these genes can be identified in L. major.

The T. brucei GDP-fucose biosynthesis enzymes are active in vitro - The presence of putative TbGMD and TbGMER genes, together with the recent detection of GDP-fucose in T. brucei (46), suggested that the protein products of these genes would be active GDP-fucose biosynthetic enzymes. To assess this, we expressed both the TbGMD and TbGMER genes in E. coli with the addition of N-terminal GST tags. In both cases, soluble protein could be purified by GST affinity chromatography (Fig. 1A). The identities of the proteins were confirmed by tryptic mass fingerprinting (data not shown) and both proteins appeared relatively pure, apart from a minor contaminating band of GST at 26 kDa. The enzymes were assayed as described in Experimental Procedures, the sugar nucleotides produced were purified by solid phase extraction and analysed by LC-MS/MS (46). When both enzymes were added to the reaction, GDP-fucose was detected, indicating that these enzymes are indeed active in vitro (Fig. 1B, top panel). When only the TbGMER enzyme is added, there is no turnover of GDP-mannose to GDP-fucose (Fig. 1B, middle panel). Interestingly, when only the TbGMD enzyme was added to the reaction mixture, there was turnover of GDP-mannose to a novel GDP-deoxyhexose (Fig. 1B, bottom panel). The possible identity of this novel GDP-deoxyhexose is discussed later.

Localisation of TbGMD - To assess the subcellular location of the TbGMD protein, an HA tagged version of the gene was introduced to the ribosomal DNA locus of procyclic form T. brucei using the pLew82 expression vector (42). Successful expression was monitored by Western blotting with anti-HA antibodies and by sugar nucleotide analysis, which showed roughly a 4-fold increase in GDP-fucose and a large increase in the aforementioned novel GDP-deoxyhexose (data not shown). Immunofluorescence microscopy using anti-HA antibodies produced a series of discrete spots, suggesting that the protein might be glycosomal (Fig. 2, bottom left panel). To address this we also stained the cells using anti-GAPDH antibody, a commonly used glycosomal marker (48, 49) (Fig. 2, bottom right). The merged image shows a significant degree of co-localisation indicating that the expressed HA tagged TbGMD is localised in the glycosomes in procyclic form cells (Fig. 2, top right).
Construction of TbGMD conditional null mutant cell lines - To ensure that TbGMD was suitable for gene replacement, we performed a Southern blot to determine copy number per haploid genome. With most of the restriction enzyme digestions, TbGMD appeared as a single band, suggesting that it is present as a single copy per haploid genome (Fig. 3A). Two bands are seen with Xho I digestion, consistent with the presence of a Xho I restriction site in the TbGMD ORF.

To determine in which lifecycle stage(s) to make a conditional null mutant(s), we analysed expression of TbGMD by RT-PCR. TbGMD mRNA was detected in both bloodstream form and procyclic form lifecycle stages and bands were not seen in the absence of reverse transcriptase (Fig. 3B). This suggested that GDP-fucose might have roles in both bloodstream and procyclic form parasites and so we decided to construct conditional null mutants in both of these lifecycle stages to determine the function of GDP-fucose in T. brucei.

In the procyclic form it was possible to obtain the conditional null mutant using a conventional mutagenesis approach, i.e. replacement of one allele followed by introduction of tetracycline-inducible ectopic copy and then replacement of the second allele (7,15,50). However, using the same constructs, we failed to obtain even a single allele replacement in bloodstream form T. brucei. We presume that we were unable to obtain puromycin- or hygromycin resistant clones by simple gene replacement because transcription of the TbGMD locus in bloodstream form trypanosomes is insufficient to yield enough puromycin acetyltransferase or hygromycin phosphotransferase to provide effective drug resistance during selection. To circumvent this, we replaced the TbGMD gene with a PAC and HYG genes between T7 promoter and two T7 terminator sequences. The cell line we use constitutively expresses T7 polymerase (42) and thus we expect that much higher levels of PAC and HYG transcripts and PAC and HYG protein could be achieved using this approach, allowing us to create the conditional null mutant in bloodstream form T. brucei. A schematic representation of the creation of the conditional null mutant clones is shown in (Fig. 4A). Genomic DNA from mutant clones was analysed by Southern blotting using a TbGMD ORF probe, which showed the loss of endogenous TbGMD alleles and successful introduction of the ectopic copy in both lifecycle stages (Fig. 4B). Removal of tetracycline from the conditional mutant cells for 20 h resulted in loss of TbGMD mRNA in both the bloodstream form and procyclic form cells, as measured by RT-PCR (Fig. 4C, lanes 6-10). This confirmed that we had constructed a true conditional null mutant and allowed us to study the TbGMD mutant phenotype by growing the cells in the absence of tetracycline. RT-PCR in the absence of reverse transcriptase confirmed that there was no contaminating DNA (Fig. 4C, lanes 11-15) while RT-PCR with primers of a constitutively expressed gene, TbDPMS, confirmed that RNA was present in all reactions (Fig. 4C, lanes 1-5).

Loss of TbGMD expression results in depletion of GDP-fucose - To assess the effect of TbGMD mutation on sugar nucleotide levels, we extracted sugar nucleotides at several time points after the removal of tetracycline from the conditional null mutants. In the bloodstream form, cells grown in the presence of tetracycline showed close to wild type levels of GDP-fucose (Fig. 5A and Table 1). Growth without tetracycline for two days resulted in depletion to <10% of wild type GDP-fucose levels (Fig. 5A and Table I). In the procyclic form of the parasite, growth in the presence of tetracycline resulted in a two- to three-fold increase in the levels of GDP-fucose when compared to wild type (Fig. 5B and Table 1). This meant that six days growth without tetracycline were required before GDP-fucose levels dropped to <10% of wild type levels in the procyclic form TbGMD conditional null mutant (Table 1). In both lifecycle stages, a novel GDP-deoxyhexose
peak was detected in tetracycline-induced cells. Based upon relative retention time, we believe that this is the same peak that was observed with in the in vitro assay with TbGMD alone (Fig. 1B).

**Abrogation of TbGMD expression affects the growth rate of T. brucei** - To assess any potential growth defects in our conditional null mutant cell lines under non-permissive conditions, we grew cells in the presence and absence of tetracycline and measured their growth rates by counting cells every day. The bloodstream form mutants grew at the same rate for three days with and without tetracycline. However on day 4, the cells without tetracycline showed slower growth and subsequently started to die (Fig. 6A). These cultures resumed normal growth at day 8 / day 9 and continued to grow at the same rate as the plus tetracycline controls. RT-PCR performed with mRNA from day 10 cells showed that these cells were re-expressing the TbGMD ORF (data not shown). This kind of escape from tetracycline control of gene expression is a common feature of conditional null mutants of essential genes in bloodstream form T. brucei (7,15). The procyclic form mutants grew at the same rate for the first 7 days with and without tetracycline (Fig. 6B). On day 8 and day 9, the cells grown in the absence of tetracycline exhibited reduced growth rates and from day 10 onwards the cells started to die. In contrast, the cells grown with tetracycline induction continued to grow at a normal rate. These data indicated that TbGMD is required for growth in both of these lifecycle stages.

**Morphology of TbGMD conditional null mutants under non-permissive conditions** – We analysed cells grown with and without tetracycline using scanning electron microscopy (SEM). In the bloodstream form mutants, cells grown with or without tetracycline for four days showed an essentially normal morphology (Fig. 7A). However, cells grown without tetracycline for six days appeared predominantly as lysed cells, together with a small percentage of cells (~20%) which were aberrantly shaped and apparently lacked flagella (Fig. 7A). These aberrant cells were observed in two independent experiments between day 4 and day 6 after tetracycline removal.

In the procyclic form mutants, cells grown with or without tetracycline for 8 days showed essentially normal morphology (Fig. 7B). However, after 12 days growth in the absence of tetracycline the majority of the cells possessed detached flagella. Despite the detachment of the flagellum, the flagellar connector (51) can be seen in one of the images, suggesting that this structure is probably not affected in these mutants.

**DISCUSSION**

*T. brucei* contains active GDP-fucose biosynthesis enzymes - A recent analysis of sugar nucleotide levels in *T. brucei* provided the first evidence that this organism produces the sugar nucleotide GDP-fucose and, presumably, synthesises fucose-containing glycoconjugates (46). In this work, we have successfully identified *T. brucei* homologues of de novo GDP-fucose biosynthesis enzymes and demonstrated their ability to convert GDP-mannose to GDP-fucose in vitro. Both the incubation of GDP-mannose with TbGMD alone in vitro (supplemented with NADPH) and over-expression of TbGMD in vivo, resulted in the appearance of a novel sugar nucleotide peak in the GDP-deoxyhexose channel of the LC-MS/MS chromatogram. The specificity of the MS method employed in this work means that this peak must have the same mass as GDP-fucose, i.e. it is also a GDP-deoxyhexose. The most likely candidates for such a GDP-deoxyhexose are either GDP-rhamnose or GDP-deoxytalose, both of which can be synthesised from GDP-mannose via a GMD enzyme and appropriate reductase or epimerase/reductase (52,53). Based upon relative retention times in this type of HPLC system (52,53), we believe that our novel GDP-deoxyhexose is more likely to be GDP-rhamnose rather than GDP-deoxytalose. In
support of this hypothesis, there is a precedent in the literature for a GMD enzyme being able to synthesize GDP-rhamnose from GDP-mannose when provided with NADPH (54). We assume that over-expression of TbGMD in cells produces a situation where the TbGMER enzyme becomes rate-limiting, allowing TbGMD to reduce back the keto intermediate to GDP-rhamnose. The higher levels of the novel product in the procyclic form may simply be due to a higher level of over expression in that lifecycle stage. The novel GDP-deoxyhexose is only present at trace levels in wild type cells (46) suggesting that it does not normally contribute to the sugar nucleotide pool of T. brucei.

Loss of GDP-fucose results in growth defects and morphological changes -The almost complete loss of GDP-fucose from cells in our conditional TbGMD null mutants under non-permissive conditions suggests that the de novo biosynthesis pathway is the only source of GDP-fucose in T. brucei. This is consistent with the apparent absence genes encoding enzymes involved in the GDP-fucose salvage pathway in the T. brucei genome. L.major, which lacks obvious homologues of the de novo pathway enzymes, probably makes fucose via a salvage pathway using its GDP-arabinose biosynthesis enzymes (46,55).

The morphology of the procyclic form TbGMD mutant under non-permissive conditions has revealed one of the functions of fucose in T. brucei. The flagellar detachment is highly reminiscent of the RNAi phenotype obtained for the glycoprotein Fla-1 in procyclic form cells (41). This is not entirely unexpected, as the T. cruzi homolog of Fla-1, gp72, is known to contain fucose in its glycan chains (33-35) and T. cruzi epimastigote cells null for gp72 also show flagellar detachment (36-38). Thus, flagellar detachment in our mutant is consistent with the glycan part of Fla-1 contributing to flagellar attachment in the procyclic form, possibly through carbohydrate : carbohydrate interactions, as seen for the fucose containing LewisX structures (56) or, alternatively, through a carbohydrate : lectin interaction. Interestingly, unlike the Fla-1 RNAi experiments in bloodstream form T. brucei, there was no visible flagellar detachment or obvious effect on cytokinesis in the bloodstream form TbGMD mutant prior to cell death. One possible reason for this could be compensation by the bloodstream form specific Fla-2 glycoprotein (41). Given the strong growth phenotype that we observed in both lifecycle stages, additional roles for GDP-fucose and fucosylated glycans in this organism can not be ruled out.

Further studies to identify fucosylated glycoproteins in T. brucei will be needed to learn more about the role of this sugar in this organism. Unfortunately, conventional routes to identify fucose-containing glycoconjugates by biosynthetic labelling with ['H]fucose are not feasible because there is no apparent GDP-fucose salvage pathway in T. brucei. Furthermore, our attempts to identify α-fucosylated glycoconjugates with Ulex europaeus and Aleuria aurantia lectin blotting experiments have failed thus far. This may be because the fucose in T. brucei is in beta, rather than alpha anomeric configuration and/or because fucose residues are internal rather than terminal.

Despite the enigma of the ultimate fate(s) of fucose in T. brucei, our TbGMD conditional null mutants have shown that GDP-fucose biosynthesis, and hence fucose metabolism, are essential for bloodstream and procyclic form T. brucei. Thus, the enzymes of GDP-fucose biosynthesis and/or GDP-fucose dependent glycosyltransferases may be considered as a potential drug targets. The GDP-fucose biosynthesis enzymes themselves also represent potential targets despite high conservation between trypanosome and human enzymes because humans also possess a GDP-fucose salvage pathway that could, at least partially, compensate for inhibition of the de novo pathway (57).
TbGMD is localised in the glycosome in T. brucei - The localisation of the TbGMD enzyme in the glycosome is unexpected because in other organisms GDP-fucose biosynthesis takes place in the cytoplasm (58). It is also unexpected because the amino acid sequence of TbGMD does not contain any obvious glycosomal targeting signals. Glycosomal localisation generally depends on the presence of either a C-terminal PTS-1 tripeptide sequence (SKL or a conservative variant thereof) or an N-terminal PTS-2 sequence motif (48). In cases where no targeting sequence can be found, it has been proposed that proteins traffic to the glycosome by “piggybacking” on other glycosomally localised proteins (48). One possible partner is the second enzyme in the GDP-fucose pathway, TbGMER, which has been shown to interact with GMD in other systems (27) and which ends with the sequence ARK, where AR are PTS-1 consensus residues. It is possible that localisation to the glycosome provides a way of metabolically controlling the rate of GDP-fucose biosynthesis by sequestering the enzyme from its GDP-mannose substrate. This kind of “control by compartmentalisation” has been shown to be important in controlling enzymes involved in glycolysis in T. brucei (59). An alternative is that GDP-fucose biosynthesis occurs in the glycosome because GDP-mannose synthesis also occurs there. In this regard, it is worth noting that both T. brucei phosphomannose isomerase and phosphomannomutase have putative PTS-1 sequences at their C-termini (48).

REFERENCES


FOOTNOTES

*The authors would like to thank Drs Guther and Urbaniak for assistance with molecular biology and for useful discussions, Dr Martin for assistance with light microscopy and Martin Keirans (CHIPS facility, University of Dundee) for assistance with electron microscopy. We are grateful to Dr Oppendoes (Catholic University of Louvain) for providing the anti-GAPDH anti-serum. This work was supported by a Wellcome Trust studentship to DCT, a Marie Curie post-doctoral fellowship to LI and by a Wellcome Trust programme grant (071463) to MAJF.

The abbreviations used in this work are GMD, GDP-mannose 4,6 dehydratase; GMER, GDP-4-keto-6-deoxymannose epimerase reductase, PBS, Phosphate buffered saline; LC-MS/MS, high performance liquid chromatography tandem mass spectrometry; ER, endoplasmic reticulum.
FIGURE LEGENDS

Fig. 1. Expression and assay of *T. brucei* GDP-fucose biosynthetic enzymes. (A) Coomassie blue stained SDS-PAGE gel of purified, GST-tagged, recombinantly expressed TbGMD and TbGMER proteins. (B) The purified recombinant enzymes were assayed using GDP-mannose as a substrate. Sugar nucleotides were purified from the assay mixture using EnviCarb columns and analysed by multiple reaction monitoring LC-MS/MS (46).

Fig. 2. Localization of TbGMD-HA by immunofluorescence microscopy. Procyclic form *T. brucei* cells expressing HA tagged TbGMD were fixed and stained with rat anti-HA and FITC-conjugated anti-rat secondary antibody (bottom left), rabbit anti-GAPDH and TRITC-conjugated anti-rabbit secondary antibody (bottom right panel). The merged image (top right) and phase contrast image (top left) are also shown.

Fig. 3. TbGMD is a single copy gene expressed in both lifecycle stages. (A) *T. brucei* gDNA (~1 µg) was digested with restriction enzymes, separated by agarose gel electrophoresis and transferred to a nylon membrane. The blot was probed with a ~500 bp labeled *TbGMD* ORF probe. (B) RNA was extracted from mid log bloodstream form and procyclic form *T. brucei* and either treated (cDNA) or left untreated (RNA) with reverse transcriptase and used as a template for PCR amplification with *TbGMD* ORF primers. The PCR products were then separated on an agarose gel.

Fig. 4. Construction of bloodstream form and procyclic form *T. brucei* TbGMD conditional null mutants. (A) Constructs used in obtaining *TbGMD* conditional null mutants. Bloodstream form (bsf) constructs contain an ectopic T7 promoter and two T7 terminators to obtain sufficient transcription of drug resistance genes. Genomic DNA (1 µg) from the wild type cell line, the bloodstream form *TbGMD* conditional null mutant (*TbGMD*\(^{1/2}\)ATbGMD::HYG/\(\Delta\)TbGMD::PAC) and the procyclic form *TbGMD* conditional null mutant (*TbGMD*\(^{1/2}\)ATbGMD::BSD/\(\Delta\)TbGMD::PAC) was digested with Eco RV and separated on a 0.8% agarose gel. The DNA was transferred to a positively charged nylon membrane and the blot was hybridised using a 500 bp fluorescein labelled *TbGMD* ORF probe. The arrows indicate the bands corresponding to the endogenous (End) and ectopic (Ect) copies of the *TbGMD* gene. (C) RNA was obtained from 5 x 10\(^6\) cells from wild type cells (WT, lanes 1, 6 and 11), the bloodstream form tetracycline-induced *TbGMD* conditional null mutant (lanes 2, 7 and 12), bloodstream form *TbGMD* conditional null mutant grown without tetracycline for 20 h (lanes 3, 8 and 13), procyclic form tetracycline-induced *TbGMD* conditional null mutants (lanes 4, 9 and 14) and procyclic form *TbGMD* conditional null mutants grown without tetracycline for 20 h (lanes 5, 10 and 15). The RNA was reverse transcribed and amplified by PCR using either dolichol phosphate mannose synthetase (*TbDPMS*, lanes 1-5) primers as a positive control for the presence of mRNA or with *TbGMD* specific primers (*TbGMD*, lanes 6-10). RNA not treated with reverse transcriptase (*TbGMD* - no RT, lanes 11-15) was used as a negative control.
Fig. 5. Levels of GDP-mannose and GDP-fucose in bloodstream and procyclic form *T. brucei TbGMD* conditional null mutants under permissive and non-permissive conditions. (A) Bloodstream form cells were grown in HMI-9 with (+tet) or without (-tet) tetracycline for 2 days and sugar nucleotides were extracted from ~1 x 10^8 cells and analyzed by LC-MS/MS. Total ion count peak areas are displayed above each peak. GDP-deoxyhexose peaks were amplified as indicated in the figure. (B) As above for procyclic form cells grown in SDM-79 with or without tetracycline for 6 days. Sugar nucleotides were extracted from ~5 x 10^7 cells.

Fig. 6. Growth characteristics of bloodstream and procyclic form *T. brucei TbGMD* conditional null mutants under permissive and non-permissive conditions. Bloodstream form (A) and procyclic form (B) parasites were grown in normal HMI-9 or SDM-79 medium respectively, plus selection antibiotics, and either with or without the addition of tetracycline. Cells were counted every 24 h and diluted when appropriate. Cells were counted in triplicate and mean values ± SD are shown.

Fig. 7. Scanning electron microscopy of bloodstream and procyclic form *T. brucei TbGMD* conditional null mutants under permissive and non-permissive conditions. Conditional null mutant bloodstream form (A) and procyclic form (B) cells were grown in the presence or absence of tetracycline for the times indicated, fixed with 2.5% glutaraldehyde and prepared for SEM. The flagellar connector region (FC) is indicated by a white arrow.
Table I. Sugar nucleotide contents of bloodstream and procyclic form *TbGMD* conditional null mutants under permissive and non-permissive conditions.

<table>
<thead>
<tr>
<th>Sugar Nucleotide</th>
<th>Bloodstream form cells</th>
<th>Procyclic form cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plus tetracycline day 2</td>
<td>No tetracycline day 2</td>
</tr>
<tr>
<td></td>
<td>Plus tetracycline day 6</td>
<td>No tetracycline day 6</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>216</td>
<td>132</td>
</tr>
<tr>
<td>UDP-galactose</td>
<td>72</td>
<td>51</td>
</tr>
<tr>
<td>UDP-N-acetyl glucosamine</td>
<td>110</td>
<td>57</td>
</tr>
<tr>
<td>GDP-mannose</td>
<td>3.6</td>
<td>4.1</td>
</tr>
<tr>
<td>GDP-fucose</td>
<td>0.12</td>
<td>0.008</td>
</tr>
<tr>
<td>GDP-deoxyhexose</td>
<td>0.22</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are in pmoles per 10^7 cells. Sugar nucleotides levels were estimated using an internal standard of GDP-glucose and empirically determined molar relative response factors, as described in (46). The molar relative response factor of the novel GDP-deoxyhexose was assumed to be the same as for GDP-fucose.
GMER GMD

A

GMER = GDP-fucose synthetase
GMD = GDP-mannose dehydratase

B

Both Enzymes

GMER only

GMD only

Substrate

Product

Substrate

Product

Substrate

Novel product

Time in minutes

kDa

GMER GMD

> 96 kDa

> 66 kDa

> 45 kDa

> 30 kDa

5.00 10.00 15.00 20.00 25.00 30.00 35.00 40.00

24.27

32.55

24.73

18.85

96

66

45

30
A) BamHI, EcoRI, PstI, SacI, SalI, XhoI

B) cDNA
- Bloodstream 1 µg
- Procyclic 1 µg

RNA
- Bloodstream 4 µg
- Procyclic 4 µg

Marker
- 500 bp
- 1500 bp

Restriction
- 10 kb
- 8 kb
- 6 kb
- 5 kb
- 4 kb
- 3 kb
- 2 kb
- 1 kb
A) pcfl conditional null mutant

B) pcf conditional null mutant

C) TbDPMS, TbGMD, TbGMD - no RT

1. WT + tet - tet + tet - tet + tet - tet + tet - tet + tet - tet + tet - tet

marker: marker

TbDPMS
TbGMD
TbGMD - no RT

10kb
8kb
6kb
3kb
1kb

End.

Ect.
A) GDP-Man

B) GDP-Man

- tet

+ tet

GDP-deoxyhex

GDP-Fuc

Time in minutes
A) Plus tetracycline  No tetracycline

B) Plus tetracycline  No tetracycline
A  
plus tetracycline  
no tetracycline  
day 4

no tetracycline  
day 6

B  
plus tetracycline  
no tetracycline  
day 8

no tetracycline  
day 12
The de novo synthesis of GDP-fucose is essential for flagellar adhesion and cell growth in trypanosoma brucei
Daniel C. Turnock, Luis Izquierdo and Michael A. J. Ferguson

J. Biol. Chem. published online July 19, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M704742200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts