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A Gene of the β3-Glycosyltransferase Family Encodes N-Acetylglucosaminyltransferase II Function in *Trypanosoma brucei*

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The bloodstream form of the human pathogen *Trypanosoma brucei* expresses oligomannose, paucimannose, and complex N-linked glycans, including some exceptionally large poly-N-acetyllactosamine-containing structures. Despite the presence of complex N-glycans in this organism, no homologues of the canonical N-acetylglucosaminyltransferase I or II genes can be found in the *T. brucei* genome. These genes encode the activities that initiate the elaboration of the Manα1–3 and Manα1–6 arms, respectively, of the conserved trimannosyl-N-acetylbiosyl core of N-linked glycans. Previously, we identified a highly divergent *T. brucei* N-acetylglucosaminyltransferase I (*TbGnTI*) among a set of putative *T. brucei* glycosyltransferase genes belonging to the β3-glycosyltransferase superfamily (Damerow, M., Rodrigues, J. A., Wu, D., Güther, M. L., Mehlert, A., and Ferguson, M. A. (2014) *J. Biol. Chem.* 289, 9328–9339). Here, we demonstrate that TbGT15, another member of the same β3-glycosyltransferase family, encodes an equally divergent N-acetylglucosaminyltransferase II (*TbGnTII*) activity. In contrast to multicellular organisms, where GnTII activity is essential, *TbGnTII* null mutants of *T. brucei* grow in culture and are still infectious to animals. Characterization of the large poly-N-acetyllactosamine containing N-glycans of the *TbGnTII* null mutants by methylation linkage analysis suggests that, in wild-type parasites, the Manα1–6 arm of the conserved trimannosyl core may carry predominantly linear poly-N-acetyllactosamine chains, whereas the Manα1–3 arm may carry predominantly branched poly-N-acetyllactosamine chains. These results provide further detail on the structure and biosynthesis of complex N-glycans in an important human pathogen and provide a second example of the adaptation by trypanosomes of β3-glycosyltransferase family members to catalyze β1–2 glycosidic linkages.

The African trypanosomes are protozoan parasites that cause human African sleeping sickness and Nagana in cattle. The parasite undergoes a complex life cycle between the mammalian host and the blood-feeding tsetse fly vector (*Glossina* sp.). Throughout this life cycle, *Trypanosoma brucei* is coated by glycosylphosphatidylinositol (GPI)-anchored proteins. The bloodstream form of the parasite in the mammalian host is covered by a coat of 5 × 10⁶ variant surface glycoprotein (VSGs) homodimers and evades the immune system by replacing one VSG coat by another, in a process known as antigenic variation (1–4). The VSG GPI anchors contain side chains of 0–6 Gal residues, depending on the VSG variant (5–7) and between 1 and 3 N-linked glycans. The latter can be of oligomannose, paucimannose, or complex types (6, 8, 9). *T. brucei* expresses numerous other GPI-anchored and transmembrane glycoproteins at the cell surface, in the flagellar pocket, in the intra-cellular endosomal/lysosomal system, of which are life cycle stage-specific or display life cycle stage-specific glycosylation differences. For example, the transmembrane invariant surface glycoproteins ISG65 and ISG75 (10) and the GPI-anchored flagellar pocket ESAG6/ESAG7 heterodimeric transferrin receptors (11–13) are specific to the bloodstream life cycle stage, whereas the major lysosomal glycoprotein p67 is common to bloodstream and procyclic stages but contains complex N-glycans only in the bloodstream stage (14). This control of stage-specific glycosylation resides primarily at the level of oligosaccharyltransferase expression (15). Thus, in the bloodstream form of *T. brucei* both the *TbSTT3A* and *TbSTT3B* genes are expressed, and it appears that TbSTT3A co-translationally scans for glycosylation sequons in relatively acidic local

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7 The abbreviations used are: GPI, glycosylphosphatidylinositol; VSG, variant surface glycoprotein; sVSG, soluble form VSG; poly-LacNAc, poly-N-acetyllactosamine; GT, glycosyltransferase; GnT, GlcNAc transferase; PAC, puromycin acetyltransferase; HYG, hygromycin phosphotransferase; Hex, hexose; HexNAc, N-acetylhexosamine; HILIC, hydrophilic interaction liquid chromatography; PMAA, partially methylated alditol acetate; TLCK, tosyl-lysine chloromethyl ketone hydrochloride; CAZy, carbohydrate-active enzyme; bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol.
environments, transferring exclusively Man₆GlcNAc₂ that is destined to be processed to paucimannose or complex N-glycans, whereas TbSTT3B post-translationally modifies any remaining sequons with Man₉GlcNAC₂ that is destined to be processed no further than Man₆GlcNAC₂ in the conventional oligomannose series. Conversion from the oligomannose series to the complex series by the conventional mammalian-type route cannot occur because the parasite lacks a Golgi α-mannosidase II gene (16). In the procyelic form of *T. brucei*, the expression of *TbSTT3A* is repressed at both the mRNA level (15) and protein level (17), favoring the transfer of Man₆GlcNAC₂ and the predominant expression of the conventional Man₅GlcNAC₂-Man₆GlcNAC₂ oligomannose series (18).

The survival strategies of protozoan parasites frequently involve the participation of glycoconjugates. *T. 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 (19). The creation 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 form life stages (20–22). Similarly, the creation of UDP-GlcNAC pyrophosphorylase (*TbULP*) and glucosamine 6-phosphate N-acetyltransferase (*TbGNA*) conditional null mutants has shown that UDP-GlcNac is essential for bloodstream form of *T. brucei* (23, 24). From these experiments, 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 characterize the UDP-Gal- and UDP-GlcNac-dependent glycosyltransferase (GT) genes in the parasite. We previously reported a family of 21 genes with predicted amino acid sequences consistent with being UDP-sugar-dependent GTs. All 21 putative *T. brucei* GT amino acid sequences are similar to those of the mammalian β3GT family (25). The mammalian β3GT family includes Gal, Glc, glucuronic acid, GlcNAc, and GalNAc β-3 transferases, and its members contain N-terminal transmembrane domains followed by three conserved motifs as follows: (I/L)RXXXWG, (F/Y)(V/L/M)XXX-DXD, and (E/D)D(A/V)(Y/F)XGX(C/S). The comparable motifs in the *T. brucei* genes are slightly different, WG, Y(I,V,F)XKXDDD, and ED(A/V/I/L/M)(M/L)X(G/A), but nevertheless, they identify the parasite genes as belonging to the β3GT superfamily (26). One of these genes (*TbGT8*) encodes a β1–3 GlcNAc transferase and another (*TbGT3*) a β1–3 GlcNAc transferase that modifies the complex GPI anchor side chains of the procyclins (the major surface glycoproteins of the procyclic life cycle stage) (26–28). However, we recently reported that another gene (*TbGT11*) encodes a β1–2 GlcNAc transferase that performs a similar role to members of the N-acetylglucosaminyltransferase I family, in that it transfers GlcNAc in β1–2 linkage to the 6-arm of Man₁–6(Man₁–3)Manβ₁–4GlcNAcβ₁–4GlcNAc, emphasizing the highly divergent nature of the trypanosome genes involved in structurally conserved aspects of complex N-glycan biosynthesis.

**Experimental Procedures**

**Cultivation of Trypanosomes—** *T. brucei brucei* strain 427 bloodstream form parasites, expressing VSG variant 221 and transformed to stably express T7 polymerase and the tetracycline repressor protein under G418 antibiotic selection (31), were used in this study. This genetic background will be referred to as wild-type (WT). Cells were cultivated in HMI-9 medium containing 2.5 μg/ml G418 at 37 °C in a 5% CO₂ incubator as described previously (31).

**DNA and RNA Isolation and Manipulation—** Plasmid DNA was purified from *Escherichia coli* (α-select chemically competent cells, Bioline, London, UK) using Qiagen Miniprep or Maxiprep kits, as appropriate. Gel extraction and reaction clean up was performed using QIAquick kits (Qiagen). Custom oligonucleotides were obtained from Eurofins MWG Operon or the Dundee University oligonucleotide facility. *T. brucei* genomic DNA was isolated from ~2 × 10⁸ bloodstream form cells using DNAzol (Helena Biosciences, UK) by using standard methods. *T. brucei* mRNA was extracted from 1 × 10⁷ cells using RNeasy RNA extraction kit (Qiagen).

**Generation of Gene Replacement Constructs—** The 517-bp 5′- and 454-bp 3′ UTR sequences next to the *Tb4277.3.000* ORF were PCR-amplified from genomic DNA using *Pfu* DNA polymerase with primers 5′-cctggTGTGACatgctccccaaatgtg-3′ and 5′-tttatttgctttttctcaagtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt...
T. brucei N-Acetylgalcosaminyltransferase II

restriction sites under replacement of the GPldeAc insert, but retention of the sequence encoding for one HA tag, resulting in the plasmid pLEW82-TbGT15-HA3. The identity of all constructs was confirmed by sequencing.

Transformation of Bloodstream Form T. brucei—Constructs for gene replacement and ectopic expression were purified, digested with NotI to linearize, precipitated, washed with 70% ethanol, and re-dissolved in sterile water. The linearized DNA was electroporated into T. brucei bloodstream form cells (strain 427, variant 221) that were stably transformed to express T7 RNA polymerase and the tetracycline repressor protein under G418 selection. Cell culture and transformation were carried out as described previously (31–33).

Southern Blotting—Aliquots of genomic DNA isolated from 100 ml of bloodstream form T. brucei cultures (~2 × 10^8 cells) were digested with EcoRI, resolved on a 0.8% agarose gel and transferred onto a Hybond-N positively charged membrane (GE Healthcare, UK). Highly sensitive DNA probes labeled with digoxigenin-dUTP were generated using the PCR digoxigenin probe synthesis kit (Roche Applied Science) according to the manufacturer's recommendations and hybridized overnight at 42 °C. Detection was performed using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments and the chemiluminescent substrate CSPD (Roche Applied Science).

Mouse Infectivity Studies—Wild-type and TbGT15 null mutant bloodstream form trypanosomes were grown in HMI-9T media, washed in media without antibiotics, and resuspended at 5 × 10^6 cells/ml. Groups of five female BALB/c mice were used for each cell line, and 0.1 ml of the suspension above was injected intraperitoneally per animal. Infections were assessed 3 days post-infection by tail bleeding and cell counting using a Neubauer chamber in a phase contrast microscope.

Semi-quantitative RT-PCR—To assess the amount of Tb427.7.300 mRNA in the TbGT15 conditional null mutant cells grown under permissive and non-permissive conditions, RT-PCR were performed using AccessQuick RT-PCR System (Promega) according to the manufacturer's recommendations. A TbGT15 350-bp fragment was amplified with the primer pair 5’-cactggtgatcctgtgaga-3’ and 5’-catccaagacccgccgttaaaatgg-3’. As a control to ensure similar RNA levels in both samples, primers 5’-aaggtgctgagctcttcatc-3’ and 5’-tagaaccgagccgtgcatca-3’ amplifying a 448-bp product of dolichol phosphate mannosyl synthase (Tb10.70.2610) were used.

Small Scale sVSG Isolation—Soluble form VSG (sVSG) was isolated from 100 ml of cultures containing ~2 × 10^8 bloodstream form T. brucei by a modification of the method of Cross and co-workers (34, 35) as described previously (36). Briefly, cells were chilled on ice, centrifuged at 2500 × g for 10 min, and washed in an isotonic buffer. The pellet was resuspended in 300 μl of lysis buffer (10 mM NaH2PO4 buffer, pH 8.0, containing 0.1 mM tosyllysine chloromethyl ketone hydrochloride (TLCK), 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and incubated for 5 min at 37 °C. The sample was centrifuged at 14,000 × g for 5 min, and the supernatant was applied to a 200-μl DE52 anion exchange column pre-equilibrated in 10 mM sodium phosphate buffer, pH 8.0. Elution was performed with 0.8 ml of 10 mM sodium phosphate buffer, pH 8.0, and the eluate was concentrated and dialyzed with water on a YM-10 spin concentrator (Microcon). The final sample of 50–100 μg of sVSG221 was recovered in a volume of 100 μl of water.

ES-MS Analysis of Intact sVSG—50 μg of aliquots of sVSG preparations were diluted to ~0.05 μg/μl in 50% methanol, 1% formic acid and analyzed by positive ion ES-MS on a Q-Tof 6520 instrument (Agilent). Data were collected, averaged, and processed using the maximum entropy algorithm of the MassHunter software (Agilent).

Lectin Blotting of Cell Extracts—To analyze N-glycosylation of T. brucei bloodstream form cells, ~2 × 10^6 cells were first depleted of VSG by hypotonic lysis (34, 35). For Western blot analysis, residual cell ghosts were solubilized in SDS sample buffer containing 8 M urea, boiled with DT6, separated by SDS-PAGE (~1 × 10^7 cell eq/lane) on NuPAGE bis-Tris 4–12% gradient acrylamide gels (Invitrogen) and transferred to a nitrocellulose membrane (Invitrogen). Ponceau S staining confirmed equal loading and transfer. Glycoproteins were probed with 1.7 μg/ml biotin-conjugated ricin (RCA-120, Vector Laboratories, UK) in PBS before or after pre-incubation with 10 mg/ml D-galactose and 10 μg/ml α-lactose to confirm specific ricin binding. Detection was performed using IRDye 680LT-conjugated streptavidin and the LI-COR Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NE).

Structural Analysis of the Large N-Glycan Fraction—Bloodstream form cells of wild-type and TbGT15 null mutant cells were isolated from infected rats and processed as described (19). Briefly, VSG-depleted cell ghosts of 1 × 10^7 cells eq/lane were solubilized with SDS/urea buffer followed by lectin affinity chromatography using ricin-agaroase (RCA-120, Vector Laboratories). N-Glycans from the ricin-binding glycoproteins were released with peptide:N-glycosidase F (Flavobacterium menin- gosepticum, Roche Applied Science) and applied to a Bio-Gel P-4 gel filtration column. Aliquots of eluted fractions were subjected to methanolysis, trimethylsilylation, and GC-MS monocarboxylic composition analysis (37). Fractions that eluted in the void volume of the column (the total poly-LacNac fraction, rich in Gal and GlcNac) were pooled and used for methylation linkage analysis. After permethylation, acid hydrolysis, NaBD4 reduction, and acetylation, the resulting partially methylated alditol acetates (PMAAs) were analyzed by GC-MS (Agilent) as described previously (38). Authentic glycans of Galβ1–4Glc NAcβ1–2Manα1–6Galβ1–4GlcNacβ1–2Manα1–3Manβ1–4GlcNacβ1–4GlcNAc, lacto-N-neohexaose Galβ1–4Glc NAcβ1–6(Galβ1–4GlcNacβ1–3)Galβ1–4Glc, lacto-neotetraose Galα1–4Glcβ1–3Galβ1–4GlcNAc, and Galβ1–6Gal (Dextra Laboratories, UK) were subjected to methylation linkage analysis alongside the experimental samples. Using the PMAA derivative derived from non-reducing terminal galactose residues, common to all of these structures (i.e. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-1H-galactitol), we were able to inter-relate these data and determine the total ion current molar relative response factors for the PMAAs derived from terminal-Gal, 3-O-substituted Gal, 6-O-substituted Gal 3,6-di-O-substituted Gal, 2-O-substituted Man, 3,6-di-O-substituted Man, and 4-O-substituted GlcNAc (1.59, 0.99, 0.27, 0.90, 1.20, 1.00, and 0.23, respectively). These molar relative response fac-
tor values were used to correct the peak integrations of sample PMAA total ion current chromatograms and thus provide molar ratios of the PMAAs in the methylation linkage analyses of the wild-type and TbGT15 null glycan samples.

**GntII in Vitro Activity Assay**—TbGT15 fused to a C-terminal triple HA tag was overexpressed in *T. brucei* bloodstream form cells. 1 × 10⁶ cells were lysed on ice in 25 mM Tris, pH 7.5, 100 mM NaCl, 1% Triton X-100 containing a mixture of protease inhibitors (CompleteMini, Roche Applied Science), and 0.1 mM TLCK. Expression was confirmed by SDS-PAGE and Western blotting. Briefly, 5 × 10⁶ cell eq/lane were separated on NuPAGE bis-Tris 4–12% gradient acrylamide gels (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Ponceta S staining confirmed equal loading and transfer. Detection was performed using 0.5 μg/ml rabbit anti-HA antibody (QED Bioscience Inc., San Diego) and IRDye 680LT-conjugated donkey anti-rabbit IgG and the LI-COR Odyssey infrared imaging system (LI-COR Biosciences). For the *in vitro* activity assay, TbGT15-HA3 was immunoprecipitated using anti-HA magnetic beads (Pierce) and incubated with 1 μCi of UDP-[3H]GlcNAc (specific activity of 20–40 Ci/mmol, PerkinElmer Life Sciences), 1 mM cold UDP-GlcNAc (Sigma), and 5 μg of Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc or 25 μg of α1–3,α1–6-mannosiose (both Dextra Laboratories, Reading, UK) in 50 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂ in a total volume of 50 μl. After overnight incubation under vigorous shaking at room temperature, samples were desalted via a mixed-bed ion exchange column of 100 μl of Chelex-100 (Na⁺) over 100 μl of AG50X12 (H⁺) over 200 μl of AG3X4 (OH⁻) over 100 μl of QAE-Sephadex 25 (OH⁻), all from Bio-Rad, UK, except QAE-Sephadex (Sigma). Finally, glycans were freeze-dried and re-dissolved in 20% 1-propanol, and aliquots were spotted onto silica HPTLC plates (SI-60 HPTLC, E. Merck) or 0.2 units of α1–2,3 mannosidase from *Canavalia ensiformis* (New England Biolabs) or 0.2 units of β-N-acetylglucosaminidase from *Canavalia ensiformis* (Sigma) before TLC analysis. Plates were then dried, sprayed with EN³HANCE autofluorography enhancer (EN³HANCE, PerkinElmer Life Sciences), and exposed on x-ray film at −80 °C for 1–2 days.

For mass spectrometric analysis of the reaction product, the assay was performed using 5 mM non-radioactive UDP-GlcNAc. Samples were analyzed by LC-MS using a HILIC column (Tosoh TSKgel Amide column, 1 mm × 10 cm) and a gradient of 80 to 5% acetonitrile in 0.1% formic acid at a flow rate of 50 μl/min using a TSQ Quantiva triple-trap quadrupole mass spectrometer (Thermo Fisher Scientific). For methylation linkage analysis of the product, glycans were converted to constituent monosaccharides in the form of partially methylated alditol acetates and analyzed by GC-MS as described above.

**Scanning Electron Microscopy**—To analyze bloodstream form cells by scanning electron microscopy, cells were fixed in HMI-9 medium with 2.5% glutaraldehyde. They were further processed and examined in a Philips XL30 ESEM operating at an accelerating voltage of 15 kV by the Centre for High Resolution Imaging and Processing (CHIPS) at the University of Dundee.

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**Results**

**Analysis of the TbGT15 Gene Product**—We previously characterized the biological function of three members of a family of putative UDP-sugar-dependent GTs (1, 26, 27). In this study, Tb927.7.300 was selected for functional analysis. The gene encodes for a 367-amino acid protein with a theoretical molecular mass of 43.1 kDa. Stable isotope labeling with amino acids in cell culture-based quantitative proteomic data demonstrated that the protein expression level is 15 times higher in bloodstream form parasites compared with procyclic form parasites (40).

The *T. brucei* strain that was used in this study (Lister strain 427) differs from the one that was used for the reference genome sequencing project (TREU927). However, an alignment of Tb927.7.300 and its homologue Tb427.7.300 revealed a very high similarity with only three single nucleotide polymorphisms, none of them resulting in amino acid changes. The strain 427 gene and protein product will be referred to here as *TbGT15* and *TbGT15*, respectively.

The protein sequence contains several hallmarks of Golgi apparatus glycosyltransferases. First, a membrane protein topology prediction program based on a hidden Markov model (41) designates *TbGT15* as a type II transmembrane protein. In addition, the sequence contains a DAD motif, which is generally involved in catalytic activity of known GTs (42) as well as a dibasic motif, which functions as an endoplasmic reticulum exit signal (43). Indeed, a subcellular Golgi localization of *TbGT15* was confirmed previously (29, 30).

**Creation of Bloodstream Form *TbGT15* Null and Conditional Null Mutants**—As *TbGT15* is predominantly expressed in bloodstream form parasites (40), we decided to investigate the protein function by creating null and conditional null mutants in this life cycle stage. BLAST search of the *T. brucei* genome suggested that *TbGT15* is present as a single copy per haploid genome. Both alleles were sequentially replaced by homologous recombination using PAC and HYG drug resistance cassettes as summarized in Fig. 1A. After selection on the respective antibiotics, the generation of a *TbGT15* null mutant (Δ*TbGT15*: PAC/Δ*TbGT15*: HYG) was confirmed by Southern blot using probes for the *TbGT15* ORF and 3′ UTR (Fig. 1B). To allow for a tetracycline-inducible re-expression of the gene, an ectopic copy of *TbGT15* was introduced into the rRNA locus of the null mutant background using the pLEW100 vector (31). Clones were selected on phelemycin, and the creation of this conditional null mutant (Δ*TbGT15*: PAC/Δ*TbGT15*: HYG) was confirmed by RT-PCR (Fig. 1C).

No morphological differences between the WT and *TbGT15* null mutant parasites could be ascertained by light microscopy or by scanning electron microscopy (Fig. 2A). Compared with WT cells, the *TbGT15* null mutant parasites exhibited slightly slower growth kinetics *in vitro*, and this mild growth phenotype was partially reversed in *TbGT15* conditional null cells grown under permissive conditions (Fig. 2B). In addition, no difference in its ability to infect mice could be detected for the *TbGT15* null mutant (Fig. 2C). From this we can conclude that *TbGT15* is a non-essential gene in *T. brucei* bloodstream form cells.
VSG221 from WT cells is heterogeneously glycosylated, containing a highly galactosylated GPI anchor (5), one oligomannose N-glycan at Asn-428 (Man5-9GlcNAc2), as well as small biantennary structures ranging from Man3GlcNAc2 to Gal2GlcNAc2Man3GlcNAc2 at Asn-296 (8, 16). VSG can be isolated in its sVSG form by hypotonic lysis, which results in its release by endogenous GPI-specific phospholipase C (44).

To assess differences in the glycosylation phenotype, intact sVSGs from WT and \( \text{TbGT15} \) null mutant parasites were analyzed by ES-MS in positive-ion mode (Fig. 3). VSG molecules containing a total of four or five GlcNAc residues were present at similar levels in both genotypes, but glycoforms with six GlcNAc residues were completely absent in the \( \text{TbGT15} \) null mutant (see arrows in Fig. 3B and Table 1). Bearing in mind that four GlcNAc residues are necessary for the composition of the two N-glycan N-acetylchitobiose core structures, the lack of VSG glycoforms containing six GlcNAc residues strongly indi-
To express complex N-glycans and that TbGT15 is involved in their biosynthesis.

In Vitro Functional Activity Assay—To verify that TbGT15 encodes a glycosyltransferase that is directly involved in the biosynthesis of hybrid or complex N-glycans, we performed an in vitro assay for enzymatic activity as described previously (1). Briefly, full-length TbGT15 fused to a C-terminal 3× HA epitope tag was expressed in T. brucei bloodstream form cells and immunoprecipitated using anti-HA magnetic beads. The protein was incubated with Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc as acceptor substrate and tritium-labeled UDP-[3H]GlcNAc as donor substrate. Following desalting and removal of excess UDP-[3H]GlcNAc donor by mixed-bed ion exchange, aliquots were separated by thin layer chromatography (TLC) and analyzed by fluorography. Although a control immunoprecipitation with lysate from WT cells did not result in any [3H]GlcNAc incorporation (Fig. 4A, lane 1), the sample containing TbGT15-HA3 showed a strong signal of tritium-labeled reaction product (Fig. 4A, lane 2). This demonstrates that TbGT15 is able to transfer GlcNAc to biantennary Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc core structures. It is noteworthy that the shortened substrate Manα1–6(Manα1–3)Man lacking the chitobiose core was not used as an UDP-GlcNAc acceptor by TbG15 (Fig. 4A, lane 3).

To reveal whether GlcNAc is transferred to the Manα1–3 or Manα1–6 arm of Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc, the reaction product was treated with α1–2,3-mannosidase. As seen in the subsequent TLC/autofluorography analysis, mannosidase treatment caused an increased mobility of the reaction product (compare Rf values in Fig. 4B). This suggests that the 3-Man arm was not modified by TbGT15, leaving it susceptible to exoglycosidase cleavage, and allows us to conclude that the transferred GlcNAc residue is attached to the 6-Man arm of the Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc core structure. The anomeric configuration of the newly formed linkage was determined by β-N-acetylgalcosaminidase digestion of the reaction product. In the following TLC/autofluorography analysis, the band of tritium-labeled GlcNAcManα1,GlcNAcα2 disappeared, although the amount of free [3H]GlcNAc increased, demonstrating a β-configuration (Fig. 4C).

For further characterization of the reaction product, the assay was performed using non-radioactive UDP-GlcNAc. First, HILIC-MS was performed to identify the HexNACαHex3 reaction product (Fig. 5, A and B). A subsequent methylation linkage analysis on the reaction sample by GC-MS demonstrated the presence of 1,2,5-tri-O-acetyl-(1-deutero)-3,4,6-tri-O-methyl-mannitol, originating from 2-O-substituted mannose, which reveals that TbGT15 transfers GlcNAc in a 1–2 linkage to one of the non-reducing terminal mannose residues of the trimannosyl core (Fig. 5, C and D).

Taken together, these data show that TbGT15 is the glycosyltransferase responsible for the transfer of β1–2-linked GlcNAc to the α1–6-linked α-D-mannopyranosyl residue of Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAc and can therefore be termed an N-acetylgalcosaminyltransferase type II or TbGnTII.

N-Glycosylation Phenotype of Bloodstream Form TbGT15 Mutant Parasites—To investigate the effect of TbGT15 on the glycosylation of other proteins than VSG, total glycoproteins were extracted with SDS/urea from VSG-depleted trypanosome ghosts and analyzed by lectin blotting. As reported previously for WT T. brucei, ricin (RCA-120), a lectin that predominantly binds to non-reducing terminal β-galactose residues, showed strong binding to a series of glycoproteins running between 60 and 150 kDa (Fig. 6, 1st lane). Ricin binding to glycoproteins extracted from the TbGT15 null mutants was slightly reduced, and the apparent molecular mass of all signals was marginally smaller compared with WT signals (Fig. 6, 2nd lane). Albeit subtle, these changes in blotting pattern suggest an alteration in the synthesis of the large poly-LacNAc-containing glycans of the high molecular weight invariant glycoproteins (19).

To draw structural conclusions, we decided to analyze the ricin-binding glycoprotein fraction by methylation linkage as described previously (19). Briefly, WT and TbGT15 null mutant cells were isolated, depleled of VSGs, solubilized in SDS/urea, and glycoproteins were purified by ricin affinity chromatography. N-Linked glycans were released by peptide:N-glycosidase F and further fractionated by Bio-Gel P-4 gel filtration, resulting in two main fractions as follows: one containing the relatively small mannosse-rich N-glycans, and a Gal/GlcNAc-rich high molecular mass fraction eluting at the void volume of the Bio-Gel P-4 column (the total poly-LacNAc fraction). Aliquots of these fractions were subjected to neutral monosaccharide composition analysis by GC-MS. The molar ratios of Gal/Man...
**TABLE 1**

Isoboric glycoforms of sVSG221 identified by ES-MS

The molecular masses of different glycoforms of sVSG221 were calculated according to the indicated compositions (the theoretical mass of the assigned VSG composition is shown in parentheses). The relative abundances of those glycoforms observed in Fig. 3 for sVSG preparations from sVSG of WT cells and TbGT15 null mutant cells are indicated by –, trace, +, ++, and +++ scores. NA means not applicable.

<table>
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<tr>
<th>Mass (in Da)</th>
<th>WT/TbGT15 null mutant (theoretical)</th>
<th>Protein</th>
<th>GlcN-ino-cP</th>
<th>EtNP</th>
<th>GlcNAc</th>
<th>Man +Gal</th>
<th>WT</th>
<th>TbGT15 null mutant</th>
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<td>4</td>
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<td>++</td>
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<td>4</td>
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<td>+++</td>
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<td>24</td>
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<td>51,745/NA (51,736)</td>
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<td>6</td>
<td>25</td>
<td>+</td>
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**FIGURE 4. TbGT15 in vitro functional activity assay.** Fluorographs of HPTLC plates showing the products of UDP-[3H]GlcNAc and anti-HA-conjugated magnetic bead immunoprecipitates from T. brucei expressing TbGT15-HA, incubated with Man1–3(Man1–6)Man1–4GlcNAcMan1–6(Man1–3)Man (lane 1) or (A) are shown. As a negative control, anti-HA-conjugated magnetic beads incubated with lysates from cells not expressing TbGT15-HA, were used with Man1–3(Man1–6)Man1–4GlcNAcMan1–4GlcNAcor Man1–6(Man1–3)Man (lane 1 or 3) (A) as shown. After (lane 2) treatment with α1–2,3 mannosidase. C, [3H]GlcNAcMan1, GlcNAc2 reaction product before lane 1 and after lane 2 treatment with β-N-acetylglucosaminidase.

in the total poly-LacNAc fraction for WT (19) and TbGT15 null mutant parasites were found to be similar, 14.5:1 and 12.4:1, respectively.

Subsequent GC-MS methylation linkage analysis of the total poly-LacNAc fraction revealed structural similarities but also some quantitative differences (Table 2). Thus, both WT and TbGT15 null total poly-LacNAc glycans contain 2-O-substituted Man and 3,6-di-O-substituted Man, consistent with a conventional core structure of R-2Manα1–6(R’-2Manα1–3) Manβ1–4GlcNAcβ1–4GlcNAc in all structures. Furthermore, both samples contained significant amounts of 4-O-substituted GlcNAc, indicating the presence of multiple LacNAc
repeats. However, although the numbers of terminal Gal residues were similar, there was a significant decrease in 6-O-substituted Gal and 3-O-substituted Gal residues and a concomitant increase in 3,6-di-O-substituted-Gal residues in the TbGT15 null mutant glycans. Because TbGT15 has a GnTII-type activity that initiates elaboration of the Man/H92511–6 arm of Man/H92511–6(Man/H92511–3)Man/H92521–4GlcNAc/H92521–4GlcNAc core, these data are consistent with a model where linear poly-LacNAc chains predominate on the Man/H92511–6 arm, and the Man/H92511–3 arm is occupied by both linear and branched poly-LacNAc units (Fig. 7). The increase in the number of 3,6-di-O-substituted Gal residues in the TbGT15 mutant glycans further suggests that deletion of the entire (predominantly linear) poly-LacNAc chain attached to the Man/H92511–6 arm is, to some extent, compensated by further elaboration of the (linear and branched) poly-LacNAc chain attached to the Man/H92511–6 arm.

**Discussion**

We have cloned the gene that encodes UDP-GlcNAc:α1-6-d-mannoside-B1–2-N-acetylgalcosaminyltransferase II in *T. brucei*, an enzyme that catalyzes an essential step on the route to complex N-glycans. In human patients with carbohydrate-deficient glycoprotein syndrome type II, congenital deficiency in GnTII activity is associated with severe psychomotor retardation and other multisystemic abnormalities (45, 46). In a mouse model with a homozygous null mutation in the gene encoding GnTII (*Mgat2*−/−), 99% of...
newborns die during the first postnatal week (47). These developmental defects highlight the importance of complex N-glycans in intercellular communication and signaling in multicellular organisms.

The significance of complex N-glycans in the unicellular protozoan T. brucei is less well understood. In the bloodstream form, the parasite expresses both conventional biantennary complex N-glycans and unique highly extended and branched poly-N-acetyllactosamine-containing complex N-glycans (8, 19, 48, 49). However, the T. brucei genome contains no obvious homologues of the canonical GnTI and GnTII genes that encode the β1–2GlcNAc transferases usually responsible for the initiation of complex N-glycans. In a previous study, we identified and characterized TbGnTI, the enzyme responsible for the transfer β1–2GlcNAc to the Manα1–3 arm of N-glycan core structures (1). TbGnTI showed unusual activity in that it acts on biantennary Manα3GlcNAc2 instead of triantennary Manα3GlcNAc3, the preferred acceptor substrate for vertebrate GnTI activities (50). Remarkably, the TbGnTI gene is highly divergent from the canonical GnTI gene family and, despite the fact that TbGnTI catalyzes a β1–2 linkage, it belongs to the so-called β3-glycosyltransferase superfamily (1, 26).

In this study, a reverse-genetics approach in T. brucei bloodstream form cells indicated that the deletion of TbGT15 (another trypanosome β3-glycosyltransferase superfamily member) is accompanied by the absence of complex N-glycans, as well as alterations in the biosynthesis of the giant poly-Lac-

T. brucei N-Acetylglucosaminyltransferase II

N-acetyllactosamine-containing glycans. Using a direct enzymatic assay and comprehensive product analysis, we could show that purified TbGT15 catalyzes the conversion of Manα1–6(Manα1–3)
\[\text{Manβ1–4GlcNAcβ1–4GlcNAc}\]

or, as a binding specificity control,
\[\text{Manα1–3Manβ1–4GlcNAcβ1–4GlcNAc}\] We have therefore renamed TbGT15 to TbGnTII. The conversion of Manα3GlcNAc2 demonstrates that TbGnTII works indepen-

Quantitative GC-MS methylation linkage analysis of the total poly-LacNAc fraction

The data presented here and in Ref. 1 are consistent with the model shown here whereby in the wild-type bloodstream form of T. brucei the majority of large complex N-glycans carry highly branched poly-LacNAc chains on the Manα1–6 arm. In the TbGT15 null mutant, all elaboration of the Manα1–6 arm is absent and partly compensated by an increase in the branched poly-LacNAc elaboration of the Manα1–3 arm. Note that the relative positions of branch points shown are arbitrary.

FIGURE 7. Proposed scheme for poly-LacNAc-containing N-glycans of bloodstream form trypanosomes. The data presented here and in Ref. 1 are consistent with the model shown here whereby in the wild-type bloodstream form of T. brucei the majority of large complex N-glycans carry highly branched poly-LacNAc chains on the Manα1–6 arm. In the TbGT15 null mutant, all elaboration of the Manα1–6 arm is absent and partly compensated by an increase in the branched poly-LacNAc elaboration of the Manα1–3 arm. Note that the relative positions of branch points shown are arbitrary.

TABLE 2

Quantitative GC-MS methylation linkage analysis of the total poly-LacNAc fraction

The total poly-LacNAc fraction was permethylated, hydrolyzed, reduced, and acetylated for GC-MS analysis. The resulting PMAA derivatives were identified by retention time and electron impact mass spectra. Quantification was accomplished by integration of the total ion current using molar relative response factors deduced empirically from authentic standards, as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>PMAA derivative</th>
<th>Origin</th>
<th>WT*</th>
<th>TbGT15 null mutant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Di-O-methyl-1,3,5,6-tetra-0-acetyl-1-[2H]mannitol</td>
<td>3,6-Di-O-substituted Man</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3,4,6-Tri-O-methyl-1,2,5-tri-0-acetyl-1-[2H]mannitol</td>
<td>2-O-Substituted Man</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-methyl-1,5-di-0-acetyl-1-[2H]galactitol</td>
<td>Terminal Gal</td>
<td>4.9</td>
<td>5.0</td>
</tr>
<tr>
<td>2,6,3-Tri-O-methyl-1,3,5-tri-0-acetyl-1-[2H]galactitol</td>
<td>3-O-Substituted Gal</td>
<td>2.2</td>
<td>1.7 (down 23%)</td>
</tr>
<tr>
<td>2,3,4-Tri-O-methyl-1,5,6-tri-0-acetyl-1-[2H]galactitol</td>
<td>6-O-Substituted Gal</td>
<td>23.4</td>
<td>15.9 (down 32%)</td>
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<tr>
<td>2,4-Di-O-methyl-1,3,5,6-tetra-0-acetyl-1-[2H]galactitol</td>
<td>3,6-Di-O-substituted Gal</td>
<td>3.6</td>
<td>5.0 (up 39%)</td>
</tr>
<tr>
<td>3,6-Di-O-methyl-1,4,5-tri-0-acetyl-2-N-methylacetamido-1-[2H]glucosaminol</td>
<td>4-O-substituted GlcNAc</td>
<td>24.6*</td>
<td>16.6*</td>
</tr>
</tbody>
</table>

* Molar quantities relative to 3,6-di-O-substituted Man (one per glycan) are shown.

* Values for N-acetylglucosamine derivatives are less reliable than for hexoses.
The trypansomes' complex substituting Gal. This allows us to augment our model of the O-acylated Man5GlcNAc2 and Man2Man-GlcNAc2 substituted Gal. Because of the absence of Golgi α-mannosidase II in the parasite, trypansomine structures cannot be processed to complex N-glycans, rendering biantenary Man5GlcNAc2 transferred by STT3A the only route to paucimannose and complex structures. Furthermore, the inability of TbGnTI to act on biantenary Man5GlcNAc2 (1) also means that biantenary Man5GlcNAc2 transferred by STT3A is the only possible route to pseudohybrid N-glycans (i.e., those with only one arm of the trimannosyl-core modified by GlcNAc ± additional sugars). RNAi knockdown of TbSTT3A showed that cells are viable in culture but not in mice (15). Interestingly, the deletion of TbGnTI (TbGnTI) has no effect on in vitro growth rate, and the infectivity to mice was indistinguishable from wild type (1). This suggests that the presence of pseudohybrid N-glycans with glycan extensions to the 6-arm alone are sufficient to compensate for the loss of complex N-glycans. Here, the in vitro and in vivo viability of the TbGnTI null mutant shows that the reverse is true, in that the presence of hybrid structures with extensions to the 3-arm alone compensates for the loss of complex N-glycans. However, despite extensive attempts, a double knock-out lacking both TbGnTI and TbGnTI genes could not be generated in our hands, suggesting that extension of one or other of the arms of the N-glycan trimannosyl-core is essential for the growth and infectivity of bloodstream form of *T. brucei*.

**Author Contributions**—M. D. and M. A. J. F. designed the research and wrote the paper. M. D. and F. G. performed and analyzed experiments. M. L. S. G. performed mouse infectivity studies. A. M. and L. I. assisted in the creation of the TbgT15 mutants and the isolation and GC-MS analysis of the TbgT15 mutant glycans. All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments**—Mass spectrometry was performed in the Fingerprint Proteomics Facility that is supported by Wellcome Trust Award 097945. We thank Dr. Liaqat Ali for help with LC-MS.

[References](#)


A Gene of the β3-Glycosyltransferase Family Encodes N-Acetylglucosaminyltransferase II Function in Trypanosoma brucei

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