

A Gene of the β 3-Glycosyltransferase Family Encodes N-Acetylglucosaminyltransferase II
Function in *Trypanosoma brucei*

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ABSTRACT

The bloodstream form of the human pathogen *Trypanosoma brucei* expresses oligomannose, paucimannose and complex N-linked glycans, including some exceptionally large poly-N-acetylglucosamine-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 Man α 1-3 and Man α 1-6 arms, respectively, of the conserved trimannosyl-N-acetylchitobiosyl 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 β 3-glycosyltransferase superfamily (1). Here, we demonstrate that TbGT15, another member of the same β 3-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-acetylglucosamine containing N-glycans of the *TbGnTII* null mutants by methylation linkage

analysis suggests that, in wild-type parasites, the Man α 1-6 arm of the conserved trimannosyl core may carry predominantly linear poly-N-acetylglucosamine chains whereas the Man α 1-3 arm may carry predominantly branched poly-N-acetylglucosamine 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 β 3-glycosyltransferase family members to catalyze β 1-2 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, *T. brucei* is coated by glycosylphosphatidylinositol (GPI)⁶ anchored proteins. The bloodstream form of the parasite in the mammalian host is covered by a coat of 5×10^6 variant surface glycoprotein (VSGs) homodimers and evades the immune system by replacing one VSG coat by another, in a process known as antigenic variation (2-4). The VSG GPI anchors contain sidechains 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 and in the intracellular endosomal/lysosomal system, some of which are lifecycle stage-specific or display lifecycle 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 lifecycle stage while 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 (OST) expression (15). Thus, in bloodstream form *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 environments, transferring exclusively $\text{Man}_5\text{GlcNAc}_2$ that is destined to be processed to paucimannose or complex N-glycans, while *TbSTT3B* post-translationally modifies any remaining sequons with $\text{Man}_9\text{GlcNAc}_2$ that is destined to be processed no further than $\text{Man}_5\text{GlcNAc}_2$ 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 procyclic form *T. brucei*, the expression of *TbSTT3A* is repressed at both the mRNA level (15) and protein level (17), favouring the transfer of $\text{Man}_9\text{GlcNAc}_2$ and the predominant expression of the conventional $\text{Man}_5\text{GlcNAc}_2$ - $\text{Man}_9\text{GlcNAc}_2$ 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 lifestages (20-22). Similarly, the creation of a UDP-GlcNAc pyrophosphorylase (*TbUAP*) and glucosamine 6-phosphate N-acetyltransferase (*TbGNA*) conditional null mutants has shown that UDP-GlcNAc is essential for bloodstream form *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 β 3GT family (25). The mammalian β 3GT family includes Gal, Glc, GlcA, GlcNAc and GalNAc β -3 transferases and its members contain N-terminal transmembrane domains followed by three conserved motifs of: (I/L)RXXWG, (F/Y)(V/L/M)XXXDXD 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, identify the parasite genes as belonging to the β 3GT superfamily (26). One of these genes (*TbGT8*) encodes a β 1-3 GlcNAc transferase and another (*TbGT3*) a β 1-3 Gal transferase that modify the complex GPI anchor sidechains of the procyclins (the major surface glycoproteins of the procyclic lifecycle stage) (26-28). However, we recently reported that another gene (*TbGT11*) encodes a β 1-2 GlcNAc transferase that performs a similar role to members of the N-acetylglucosaminyltransferase I family, in that it transfers GlcNAc in β 1-2 linkage to the 3-arm of $\text{Man}\alpha$ 1-6($\text{Man}\alpha$ 1-3) $\text{Man}\beta$ 1-4GlcNAc β 1-4GlcNAc (1).

Here, we report that another *T. brucei* β 3GT superfamily gene member (*TbGT15*) encodes another β 1-2 GlcNAc transferase, already localized to the Golgi apparatus (29,30), that performs a similar role to members of the N-acetylglucosaminyl transferase II family, in that it transfers GlcNAc in β 1-2 linkage to the 6-arm of

Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc, emphasizing the highly divergent nature of the trypanosome genes involved in structurally-conserved aspects of complex N-glycan biosynthesis.

EXPERIMENTAL PROCEDURES

Cultivation of Trypanosomes—*Trypanosoma 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 from hereon as wild-type (WT). Cells were cultivated in HMI-9 medium containing 2.5 μ g/ml G418 at 37 °C in a 5% CO₂ incubator as described in (31).

DNA & 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 cleanup 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 $\sim 2 \times 10^8$ bloodstream form cells using DNAzol (Helena Biosciences, UK) using standard methods. *T. brucei* mRNA was extracted from 1×10^7 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 Tb427.7.300 ORF were PCR-amplified from genomic DNA using *Pfu* DNA polymerase with primers 5'-cggttGTCGACagtatccgcaaaatgcgact-3' and 5'-gtttaaacttacggaccgtcaagctttattttctttcctacgcac-3', 5'-gacggtccgtaagtttaaaccgatccaaagcgaataaaaaataaac-3' and 5'-ataagtaaGCGGCCGCagatgtcgcgcaagaaaaac-3' as forward and reverse primers, respectively. The two PCR products were used together in a further PCR reaction to yield a product containing the 5'-UTR linked to the 3'-UTR by a short *Hind*III (underlined), *Pme*I (italics), and *Bam*HI (underlined) cloning site and *Not*I and *Sal*I

restriction sites at each end (capital letters). The product was cloned between the *Not*I and *Sal*I sites of the pGEM-5Zf(+) vector (Promega).

The hygromycin phosphotransferase (*HYG*) and puromycin acetyltransferase (*PAC*) drug-resistance genes were then introduced into the targeting vector via the *Hind*III and *Bam*HI cloning sites. For re-expression of Tb427.7.300 the ORF was PCR-amplified from genomic DNA with the primer pair 5'-agaaagcttatggtgtggagtgggcataaa-3' and 5'-ttcagatctcatgtgcacgagcgtgcca-3' and cloned into pLEW100-Phleo (31).

For overexpression of full-length TbGT15 with a C-terminal 3x HA epitope tag, a plasmid was generated based on the trypanosome expression vector pLEW82 (31). *TbGT15* ORF was amplified from *T. brucei* genomic DNA and the primers 5'-gactaagcttatggtgtggagtgggcataaaac-3' and 5'-gact taattaatgcgtaatcagggacgtcataaggatatgcgtaatcaggacgtcataaggatacgtctccgcTGTGCACGAGGC GTGCCATC-3' containing a *Hind*III and *Pac*I restriction site (underlined), respectively. The sequence encoding for two HA tags (italics) followed by a sequence encoding an Ala-Gly-Ala linker was attached as a 5' overhang of the reverse primer. The PCR product was subcloned into pLEW82-*GPIdeAc*-HA (32) via *Hind*III and *Pac*I restriction sites under replacement of the *GPIdeAc* 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 *Not*I 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 was carried out as described previously (31-33).

Southern blotting—Aliquots of genomic DNA isolated from 100 ml of bloodstream form *T. brucei* cultures ($\sim 2 \times 10^8$ cells) were digested with

EcoRI, resolved on a 0.8% agarose gel and transferred onto a Hybond-N positively charged membrane (GE Healthcare, Amersham, UK). Highly-sensitive DNA probes labelled with DIG-dUTP were generated using the PCR DIG Probe Synthesis Kit (Roche) 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).

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 5 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 three days post-infection by tail bleeding and cell counting using a Neubauer chamber in a phase contrast microscope.

Semi-quantitative RT-PCR—In order 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 reactions 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'-cacattgtcgcgggatgtgagtga-3' and 5'-ccatccaagtaccgcggtaaaatggg-3'. As a control to ensure similar RNA levels in both samples, primers 5'-aatggatcggaccttcagcaccac-3' and 5'-tagaacctgagcgcggtgccatac-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 $\sim 2 \times 10^8$ bloodstream form *T. brucei* by a modification of the method of Cross (34,35) as described in (36). Briefly, cells were chilled on ice, centrifuged at $2500 \times g$ for 10 min and washed in an isotonic buffer. The pellet was resuspended in 300 μ l of lysis buffer (10 mM NaH_2PO_4 buffer, pH 8.0, containing 0.1 mM tosyllysine chloromethyl ketone hydrochloride (TLCK), 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin) and incubated for 5 min at 37 °C. The sample was

centrifuged at $14,000 \times g$ for 5 min, and the supernatant was applied to a 200- μ l 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, the eluate was concentrated and diafiltered with water on a YM-10 spin concentrator (Microcon). The final sample of 50–100 μ g sVSG221 was recovered in a volume of 100 μ l of water.

ES-MS analysis of intact sVSG—50 μ g aliquots of sVSG preparations were diluted to ~ 0.05 μ g/ μ l 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, $\sim 2 \times 10^8$ 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 DTT, separated by SDS-PAGE (approx. 1×10^7 cell equivalents/lane) on NuPAGE bis-Tris 4–12% gradient acrylamide gels (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Ponceau S staining confirmed equal loading and transfer. Glycoproteins were probed with 1.7 μ g/ml biotin-conjugated ricin (RCA-120, Vector Laboratories, UK) in PBS before or after pre-incubation with 10 mg/ml D-galactose and 10 mg/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, US).

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^{11} cell equivalents were solubilized with SDS/urea buffer followed by lectin affinity chromatography using ricin-agarose (RCA-120, Vector Laboratories). N-glycans from the ricin-binding glycoproteins were released with PNGase-F (Flavobacterium meningosepticum, Roche) and applied to a Bio-

Gel P-4 gel filtration column. Aliquots of eluted fractions were subjected to methanolysis, trimethylsilylation and GC-MS monosaccharide 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 Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc, lacto-N-neohexaose Galβ1-4GlcNAcβ1-6(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-1[²H]galactitol), we were able to inter-relate these data and determine the total ion current molar relative response factors (MRRFs) 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 MRRF 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.

GnTIII in vitro activity assay—TbGT15 fused to a C-terminal triple HA-tag was overexpressed in *T. brucei* bloodstream form cells. 1×10⁹ cells were lysed on ice in 25 mM Tris pH 7.5; 100 mM NaCl; 1% TritonX-100 containing a cocktail of protease inhibitors (CompleteMini, Roche) and 0.1 mM TLCK. Expression was confirmed by SDS-PAGE and Western blotting. Briefly, 5×10⁶ cell equivalents/lane were separated on NuPAGE bis-Tris 4–12% gradient acrylamide gels (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Ponceau S staining confirmed equal

loading and transfer. Detection was performed using 0.5 μg/ml rabbit-anti-HA antibody (QED Bioscience Inc., San Diego, US) and IRDye 680LT conjugated donkey-anti-rabbit IgG and the LI-COR Odyssey Infrared Imaging System (LICOR Biosciences, Lincoln, US). For the *in vitro* activity assay, TbGT15-HA₃ was immunoprecipitated using anti-HA bead magnetic beads (Pierce) and incubated with 1 μCi UDP-[³H]-GlcNAc (specific activity of 20-40 Ci/mmol, Perkin Elmer, Waltham, US), 1 mM cold UDP-GlcNAc (Sigma) and 5 μg of oligomannose-3 (a) or 25 μg α1-3,α1-6 mannotriose (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 Chelex-100 (Na⁺) over 100 μl AG50X12 (H⁺) over 200 μl AG3X4 (OH⁻) over 100 μl QAE-Sephadex25 (OH⁻), all from Bio-Rad Laboratories, UK except QAE-Sephadex (Sigma). Finally, glycans were freeze-dried, re-dissolved in 20% 1-propanol and aliquots were spotted onto silica HPTLC plates (SI-60 HPTLC, Millipore) which were run twice in 1-propanol:acetone:H₂O (9:6:4). For product analysis, samples were treated with 128 units α1-2,3 mannosidase from *Xanthomonas manihotis* (NEB) or 0.2 units β-N-acetylglucosaminidase from *Canavalia ensiformis* (Sigma-Aldrich) before TLC analysis. Plates were then dried, sprayed with EN³HANCE autofluorography enhancer (EN³HANCE, Perkin Elmer, Waltham, US), 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 x 10 cm) and a gradient of 80-5% acetonitrile in 0.1% formic acid at a flow rate of 50 μl/min using a TSQ Quantiva triple-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 (SEM), 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 15kV by the Centre for High Resolution Imaging and Processing (CHIPS) at the University of Dundee.

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 (1,26,39). In the present 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. SILAC-based quantitative proteomic data demonstrated that the protein expression level is 15 times higher in bloodstream form parasites compared to 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 3 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 DXD motif, which is generally involved in catalytic activity of known GTs (42) as well as a dibasic motif, which functions as an ER 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. 1 A. After selection on the respective antibiotics, the generation of a *TbGT15* null mutant ($\Delta TbGT15::PAC/\Delta TbGT15::HYG$) 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 phleomycin and the creation of this conditional null mutant ($\Delta TbGT15^{Ti}/\Delta TbGT15::PAC/\Delta TbGT15::HYG$) was confirmed by RT-PCR (Fig. 1 C).

No morphological differences between the WT and *TbGT15* null mutant parasites could be ascertained by light microscopy or by scanning electron microscopy (Fig. 2 A). Compared to 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. 2 C). From this we can conclude that *TbGT15* is a non-essential gene in *T. brucei* bloodstream form cells.

Characterization of VSG from WT and *TbGT15* Null Mutant Parasites – VSG221 from WT cells is heterogeneously glycosylated, containing a highly galactosylated GPI anchor (5), one oligomannose N-glycan at Asn-428 ($Man_5-9GlcNAc_2$) as well as small biantennary structures ranging from $Man_3GlcNAc_2$ to $Gal_2GlcNAc_2Man_3GlcNAc_2$ at Asn-296 (8,16). VSG can be isolated in its soluble form (sVSG) by hypotonic lysis, which results in its release by of endogenous GPI-specific phospholipase C (44).

To assess differences in the glycosylation phenotype, intact sVSGs from WT and *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 *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 indicates a deficiency in biantennary complex N-glycans at Asn-296. From this we can conclude that the mutant cells are unable to express complex N-glycans and that *TbGT15* is involved in their biosynthesis.

In vitro functional activity assay – In order 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 in (1). Briefly, full length *TbGT15* fused to a C-terminal 3x HA epitope tag was expressed in *T. brucei* bloodstream form cells and immunoprecipitated using anti-HA magnetic beads. The protein was incubated with Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc as acceptor substrate and tritium-labelled 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. While a control immunoprecipitation with lysate from WT cells did not result in any [³H]GlcNAc incorporation (Fig. 4 A, lane 2), the sample containing *TbGT15*-HA₃ showed a strong signal of tritium-labelled reaction product (Fig. 4 A, lane 1). This demonstrates that *TbGT15* is able to transfer GlcNAc to biantennary Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc core structures. It is noteworthy that the shortened substrate Man α 1-6(Man α 1-3)Man lacking the chitobiose core was not used as an UDP-GlcNAc acceptor by *TbGT15* (Fig. 4, lane 3).

To reveal whether GlcNAc is transferred to the Man α 1-3 or Man α 1-6 arm of Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc, the reaction product was treated with α 1-2,3 mannosidase. As seen in the subsequent TLC/autofluorography analysis, mannosidase treatment caused an increased mobility of the reaction product (compare *R_f* values in Fig. 4 B). 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 Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc core structure. The anomeric configuration of the newly formed linkage was determined by β -N-acetylglucosaminidase digestion of the reaction product. In the following TLC/autofluorography analysis the band of tritium-labelled GlcNAcMan₃GlcNAc₂ disappeared while the amount of free [³H]GlcNAc increased, demonstrating a β -configuration (Fig. 4 C).

For further characterisation of the reaction product, the assay was performed using non-radioactive UDP-GlcNAc. First, HILIC-MS was performed to identify the HexNAc₃Hex₃ reaction product (Fig. 5 A, 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, D).

Taken together, these data show that *TbGT15* is the glycosyltransferases responsible for the transfer of β 1-2-linked GlcNAc to the α 1-6-linked α -D-mannopyranosyl residue of Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc and can, therefore, be termed an N-acetylglucosaminyltransferase 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 previously reported 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 kDa and 150 kDa (Fig. 6, lane 1). 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 to WT signals (Fig. 6, lane 2). 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).

In order to draw structural conclusions, we decided to analyze the ricin-binding glycoprotein fraction by methylation linkage as described in (19). Briefly, WT and *TbGT15* null mutant cells were isolated, depleted of VSGs, solubilized in SDS/urea and glycoproteins were purified by ricin affinity chromatography. N-linked glycans were released by PNGaseF and further fractionated by Bio-Gel P-4 gel filtration, resulting in two main factions: one containing the relatively small mannose-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 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-2Man α 1-6(R'-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc in all structures. Further, both samples contained significant amounts of 4-O-substituted GlcNAc, indicating the presence of multiple LacNAc repeats. However, whereas 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. Since *TbGT15* has a GnTIII-type activity that initiates elaboration of the Man α 1-6 arm of Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc core, these data are consistent with a model where linear poly-LacNAc chains predominate on the Man α 1-6 arm, whereas the Man α 1-3 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 α 1-6 arm is, to some extent, compensated by further elaboration of the (linear and branched) poly-LacNAc chain attached to the Man α 1-6 arm.

DISCUSSION

We have cloned the gene which encodes UDP-GlcNAc: α 1-6-D-mannoside- β 1-2-N-acetylglucosaminyltransferase 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 signalling 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-acetylglucosamine-containing complex N-glycans (8,19,48,49). However, the *T. brucei* genome contains no obvious homologous of the canonical *GnTI* and *GnTIII* genes that encode the β 1-2GlcNAc 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 β 1-2GlcNAc to the Man α 1-3 arm of N-glycan core structures (1). *TbGnTI* showed unusual activity in that it acts on biantennary Man₃GlcNAc₂ instead of triantennary Man₅GlcNAc₂, 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* catalyses a β 1-2 linkage, it belongs to the so-called β 3-glycosyltransferase superfamily (1,26).

In the current study, a reverse-genetics approach in *T. brucei* bloodstream form cells

indicated that the deletion of *TbGT15* (another trypanosome β 3-glycosyltransferase superfamily member) is accompanied by the absence of complex N-glycans, as well as alterations in the biosynthesis of the giant poly-LacNAc-containing glycans. Using a direct enzymatic assay and comprehensive product analysis, we could show that purified TbGT15 catalyzes the conversion of $\text{Man}\alpha$ 1-6($\text{Man}\alpha$ 1-3) $\text{Man}\beta$ 1-4GlcNAc β 1-4GlcNAc to $\text{GlcNAc}\beta$ 1-2 $\text{Man}\alpha$ 1-6($\text{Man}\alpha$ 1-3) $\text{Man}\beta$ 1-4GlcNAc β 1-4GlcNAc. We have therefore renamed TbGT15 to TbGnTII. The conversion of $\text{Man}_3\text{GlcNAc}_2$ demonstrates that TbGnTII works independently from prior TbGnTI action, which is in contrast to canonical GnTIIs that only use substrates after modification by GnTI, i.e. $\text{Man}\alpha$ 1-6($\text{GlcNAc}\beta$ 1-2 $\text{Man}\alpha$ 1-3) $\text{Man}\beta$ 1-4GlcNAc β 1-4GlcNAc (51,52). This unusual acceptor specificity of the trypanosome enzyme was already indicated by previous data which show the presence of “pseudohybrid” N-glycans in the absence of TbGnTI (1) and highlights the divergent nature of TbGnTII. A phylogram based on a multiple sequence alignment of TbGT15 and GnTII enzymes of other species is shown in Fig. 8. The GnTIIs of multicellular organisms belong to the CAZy (carbohydrate-active enzymes) GT family 13 (53) and are all closely related, with nearly 90% identity between the human and mouse enzymes. In contrast, TbGnTII is a member of the CAZy GT 31 family (26) and shares only 9% identity with the human sequence at the amino acid level. Interestingly, while human GnTI and GnTII proteins have only a low level of amino acid sequence homology between them (22%), the TbGnTI and TbGnTII enzymes share 42% identity. This is consistent with the closer functional similarity of the trypanosome enzymes, which both work on the same acceptor substrate ($\text{Man}_3\text{GlcNAc}_2$, though only the latter requires the N-acetyl-chitobiose core), whereas the canonical GnTI and GnTII enzymes work on triantennary $\text{Man}_5\text{GlcNAc}_2$ and $\text{Man}\alpha$ 1-6($\text{GlcNAc}\beta$ 1-2 $\text{Man}\alpha$ 1-3) $\text{Man}\beta$ 1-4GlcNAc β 1-4GlcNAc, respectively.

Methylation linkage analysis of the poly-LacNAc N-glycans of the *TbGT15* null mutant

showed a reduction in 6-O-substituted Gal and 3-O-substituted Gal but an increase in 3,6-O-substituted Gal. This allows us to augment our model of the parasites’ complex N-glycans and propose that the $\text{Man}\alpha$ 1-6 arm is normally occupied by predominantly linear poly-LacNAc repeats and the $\text{Man}\alpha$ 1-3 arm by branched as well as linear poly-LacNAc repeats (Fig. 7).

T. brucei has an unusual dual N-glycosylation mechanism with two paralogous oligosaccharyltransferases, TbSTT3A and TbSTT3B, that transfer biantennary $\text{Man}_5\text{GlcNAc}_2$ and triantennary $\text{Man}_9\text{GlcNAc}_2$, respectively, in a site-specific manner (15,16). Due to the absence of Golgi α -mannosidase II in the parasite, triantennary structures cannot be processed to complex N-glycans, rendering biantennary $\text{Man}_5\text{GlcNAc}_2$ transferred by STT3A the only route to paucimannose and complex structures. Further, the inability of TbGnTI to act on triantennary $\text{Man}_5\text{GlcNAc}_2$ (1) also means that biantennary $\text{Man}_5\text{GlcNAc}_2$ 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 $\text{GlcNAc} \pm$ additional sugars). RNAi knockdown of TbSTT3A showed that cells are viable in culture, but not in mice (15). Interestingly, the deletion of *TbGnTI* (*TbGT11*) 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 the loss of complex N-glycans. Here, the *in vitro* and *in vivo* viability of the *TbGnTII* null mutant shows that the reverse is true, in that the presence of pseudohybrid structures with extensions to the 3-arm alone compensates for the loss of complex N-glycans. However, despite extensive attempts, a double knockout lacking both *TbGnTI* and *TbGnTII* 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 *T. brucei*.

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Author contributions—MD and MAJF designed the research and wrote the paper. MD and FG performed and analyzed experiments. MLSG performed mouse infectivity studies. AM and LI 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.

FOOTNOTES

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⁶The abbreviations used are: GPI, glycosylphosphatidylinositol; VSG, variant surface glycoprotein; sVSG, soluble-form VSG; GlcNAc, N-acetylglucosamine; Man, mannose; Gal, galactose; Glc, glucose; GlcA, glucuronic acid; GalNAc, N-acetylgalactosamine; poly-LacNAc, poly-N-acetyllactosamine; GT, glycosyltransferase; GnT, GlcNAc transferase; OST, oligosaccharyltransferase; ER, endoplasmic reticulum; SILAC, stable isotope labelling with amino acids in cell culture; cKO, conditional null mutant; PAC, puromycin acetyltransferase; HYG, hygromycin phosphotransferase; Hex, hexose; HexNAc, N-acetylhexosamine; HILIC, hydrophilic interaction liquid chromatography; PMAAs, partially methylated alditol acetates; TLCK, tosyllysine chloromethyl ketone hydrochloride; CAZy, carbohydrate-active enzymes.

FIGURE LEGENDS

FIGURE 1. Generation of a bloodstream form *TbGT15* null and conditional null mutant. A, Gene replacement strategy to create *TbGT15* null mutant cells and subsequent insertion of tetracycline-inducible ectopic copy, in brackets, to create a conditional null mutant. B, Southern blot of genomic DNA digested with *EcoRI* from WT (lanes 1, 3, 5) and *TbGT15* null mutant cells (lanes 2, 4, 6). The blot was probed with a *TbGT15* ORF probe (left-hand panel) and a *TbGT15* 3'UTR probe (middle panel) and shows the replacement of both alleles with drug resistance cassettes. Equal loading was verified by ethidium bromide staining (right-hand panel). C, Ethidium bromide-stained agarose gel of reverse transcription-PCR products from RNA extracted from WT cell, *TbGT15* null and conditional null mutants: *TbGT15* mRNA was detected in WT (lane 1) and *TbGT15* conditional null mutant cells grown under permissive (plus tetracycline) conditions (lane 4), while no mRNA was found in *TbGT15* null mutants (lane 2) and *TbGT15* conditional null mutants grown under non-permissive conditions (lane 5). To show equal RNA input, a control using dolichol-phosphate mannosyl transferase (*DPMS*) primers was performed with *TbGT15* null mutants (lane 3) and *TbGT15* conditional null mutants grown under non-permissive conditions (lane 6).

FIGURE 2. The absence of *TbGT15* does not affect bloodstream form trypanosome morphology and growth. A, Scanning electron micrographs of representative WT (left-hand panel) and *TbGT15* null mutant cells (right-hand panel); scale bar = 2 μ m. B, Growth curves for bloodstream form *T. brucei* wild-type cells (WT; grey diamonds), *TbGT15* null mutant cells (*TbGT15*DKO; black squares) and *TbGT15* conditional null mutant cells grown under permissive conditions (*TbGT15*cDKO; grey triangles). Cell counts were taken daily in triplicate, error bars indicate one standard deviation of the mean. C, infectivity of wild-type and *TbGT15* null mutant bloodstream-form parasites to mice. Mice were infected with 5×10^5 cells of WT (black) or *TbGT15* null mutants (grey) and the number of parasites per ml blood was counted three days post-infection. No difference in infectivity was observed.

FIGURE 3. sVSG221 glycoform analysis by ES-MS. Samples of intact sVSG of WT (A) and *TbGT15* null mutant trypanosomes (B) were analyzed by positive-ion ES-MS and the deconvolved spectra of the various isobaric glycoforms were generated (the compositions of these glycoforms are given in Table 1). Significant differences in the sVSG glycosylation patterns are indicated by arrows in panel B.

FIGURE 4. *TbGT15* in vitro functional activity assay. Fluorographs of HPTLC plates showing the products of: A, UDP-[3 H]GlcNAc and anti-HA-conjugated magnetic bead immunoprecipitates from *T. brucei* expressing *TbGT15*-HA₃ incubated with Man α 1-3(Man α 1-6)Man β 1-4GlcNAc β 1-4GlcNAc or Man α 1-6(Man α 1-3)Man (lane 1 or 3). As a negative control, anti-HA-conjugated magnetic beads incubated with lysates from cells not expressing *TbGT15*-HA₃ were used with Man α 1-3(Man α 1-6)Man β 1-4GlcNAc β 1-4GlcNAc (lane 2). B, The [3 H]GlcNAcMan₃GlcNAc₂ reaction product before (lane 1) and after (lane 2) treatment with α 1-2,3 mannosidase. C, The [3 H]GlcNAcMan₃GlcNAc₂ reaction product before (lane 1) and after (lane 2) treatment with β -N-acetylglucosaminidase.

FIGURE 5. Mass spectrometry analysis of *TbGT15* reaction product. HILIC-MS of *in vitro* assay reaction. A, Base peak chromatogram showing substrate HexNAc₂Hex₃ (13.36 min) and reaction product

HexNAc₃Hex₃ (13.57 min). *B*, Mass spectrum of HexNAc₂Hex₃ (substrate, S) and HexNAc₃Hex₃ (product, P) in protonated and sodiated form ([S+H]⁺=911.51; [S+Na]⁺= 933.43; [P+H]⁺=1114.58; [P+Na]⁺=1136.55). *C*, GC-MS extracted ion chromatogram of ions (*m/z* 102, 118, 129, 117, 161, and 190) characteristic for partially methylated alditol acetate (PMAA) derivatives obtained after permethylation, acid hydrolysis, sodium borodeuteride reduction, and peracetylation of the TbGT15 reaction product. *D*, spectrum of the 1,2,5-tri-O-acetyl-(1-deutero)-3,4,6-tri-O-methyl-mannitol PMAA derived from 2-O-substituted mannose, with characteristic fragment ion assignments.

FIGURE 6. Lectin blotting of VSG-depleted glycoproteins. Lysates of washed WT or *TbGT15* null mutant (KO) trypanosome cell ghosts were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with ricin (*left-hand panel*) or, as a binding specificity control, with ricin that was pre-incubating with 10 mg/ml each of galactose and lactose (*middle panel*). Equal loading and transfer is demonstrated by Ponceau S staining (*right-hand panel*).

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

FIGURE 8. Phylogenetic tree of GnTII amino acid sequences from different species. Amino acid sequences were aligned using the COBALT constraint-based multiple alignment program. GnTII: *Oryza sativa* (NP_001048400.2), *Caenorhabditis elegans* (NP_505864.1), *Drosophila melanogaster* (NP_651763.4), *Anopheles gambiae* (XP_313681.5) *Danio rerio* (NP_001077344.1), *Xenopus tropicalis* (NP_001006759.1), *Homo sapiens* (NP_002399.1), *Mus musculus* (NP_666147.1), *Rattus norvegicus* (NP_446056.1). The evolutionary distance is represented by the length of the horizontal lines.

TABLES

MW [Da] WT/ <i>TbGT15</i> (Theo.)	WT	mutant	Protein ^a	GlcN- Ino-cP ^b	EtNP	GlcNAc	Man+Gal	WT	<i>TbGT15</i> null mutant
50204/50202 (50194)	1		1	1	1	4	16	+ ^c	Traces
50241/50242 (50235)	1		1	1	1	5	17	Traces	+
50366/50364 (50356)	1		1	1	1	4	17	+	+
50405/50404 (50397)	1		1	1	1	5	16	Traces	+
50528/50526 (50518)	1		1	1	1	4	18	+++	++
50568/50566 (50559)	1		1	1	1	5	17	+	Traces
50608/NA (50600)	1		1	1	1	6	16	Traces	-
50690/50688 (50680)	1		1	1	1	4	19	+++	+++
50731/50728 (50721)	1		1	1	1	5	18	+	+
50770/NA (50762)	1		1	1	1	6	17	Traces	-
50852/50850 (50842) ^d	1		1	1	1	4	20	+++	+++
50893/50891 (50883)	1		1	1	1	5	19	++	+
50933/NA (50924)	1		1	1	1	6	18	+	-
51014/51012 (51004)	1		1	1	1	4	21	++	+++
51055/51053 (51045)	1		1	1	1	5	20	+++	+
51097/NA (51086)	1		1	1	1	6	19	+	-
51177/51175 (51166)	1		1	1	1	4	22	+	+
51217/51216 (51207)	1		1	1	1	5	21	++	+
51258/NA (51248)	1		1	1	1	6	20	+	-
51340/51337 (51328)	1		1	1	1	4	23	+	+
51380/51377 (51369)	1		1	1	1	5	22	+	Traces
51421/NA (51410)	1		1	1	1	6	21	+	-
51502/51500 (51490)	1		1	1	1	4	24	+	+
51542/51539 (51531)	1		1	1	1	5	23	+	Traces
51583/NA (51572)	1		1	1	1	6	22	+	-
51665/51662 (51652)	1		1	1	1	4	25	+	Traces
51706/51704 (51693)	1		1	1	1	5	24	+	Traces
51745/NA (51734)	1		1	1	1	6	23	Traces	-

TABLE 1. Isobaric glycoforms of sVSG221 identified by ES-MS. The molecular weights of different glycoforms of sVSG221 were calculated according to the indicated compositions (in brackets is the theoretical mass of the assigned VSG composition). 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.

^a Protein Mr is based on the amino acid sequences of the VSG221 precursor (accession no. P26332) minus residues 1-27 (signal peptide) and 460-476 (GPI attachment signal peptide) and allows for four disulphide bonds (M_r=46284).

^b Components specific to the GPI anchor and common to all glycoforms: GlcN-Ino-cP, glucosamine- α 1-6-*myo*-inositol-1,2 cyclic phosphate; EtNP, ethanolamine phosphate.

^c Maximum entropy deconvolved spectra are only semi-quantitative; an indication of relative abundance of the isobaric glycoforms is given based on peak height.

^d The most abundant glycoform of WT sVSG221 is expected to contain a GPI anchor of composition of Man₃Gal₅ (5), a C-terminal N-linked glycan of Man₉GlcNAc₂, and an internal N-linked glycan of Man₃GlcNAc₂ (8) (i.e. GlcNAc = 4, and Man = 20).

PMAA Derivative	Origin	WT ^a	<i>TbGT15</i> null mutant ^a
2,4-Di- <i>O</i> -methyl-1,3,5,6-tetra- <i>O</i> -acetyl-1-[² H]mannitol	3,6-di- <i>O</i> -substituted Man	1.0	1.0
3,4,6-Tri- <i>O</i> -methyl-1,2,5-tri- <i>O</i> -acetyl-1-[² H]mannitol	2- <i>O</i> -substituted Man	2.3	2.3
2,3,4,6-Tetra- <i>O</i> -methyl-1,5-di- <i>O</i> -acetyl-1-[² H]galactitol	Terminal Gal	4.9	5.0
2,4,6-Tri- <i>O</i> -methyl-1,3,5-tri- <i>O</i> -acetyl-1-[² H]galactitol	3- <i>O</i> -substituted Gal	2.2	1.7 (down 23%)
2,3,4-Tri- <i>O</i> -methyl-1,5,6-tri- <i>O</i> -acetyl-1-[² H]galactitol	6- <i>O</i> -substituted Gal	23.4	15.9 (down 32%)
2,4-Di- <i>O</i> -methyl-1,3,5,6-tetra- <i>O</i> -acetyl-1-[² H]galactitol	3,6-di- <i>O</i> -substituted Gal	3.6	5.0 (up 39%)
3,6-Di- <i>O</i> -methyl-1,4,5-tri- <i>O</i> -acetyl-2- <i>N</i> -methylacetamido-1-[² H]glucosaminitol	4- <i>O</i> -substituted GlcNAc	24.6 ^b	16.6 ^b

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 partially methylated alditol acetate (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 in *Experimental Procedures*.

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

^b Values for N-acetylglucosamine derivatives are less reliable than for hexoses.

Figure 1

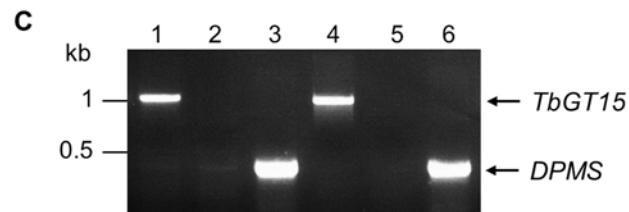
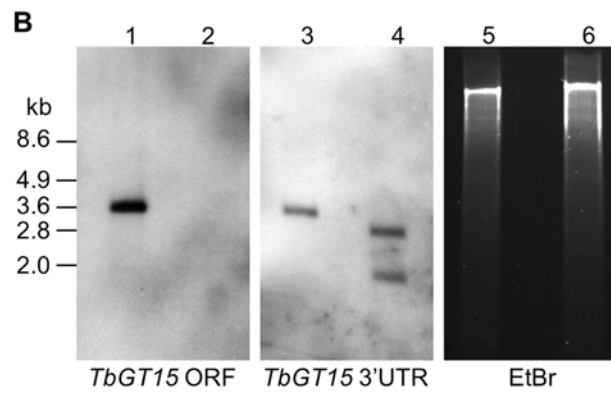
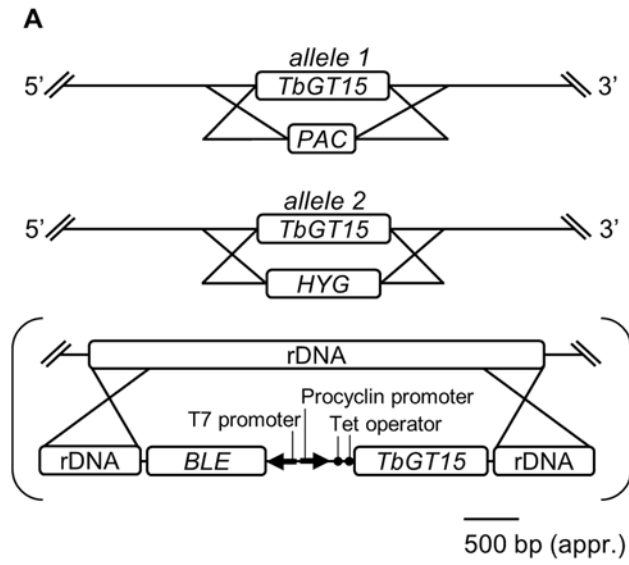


Figure 2

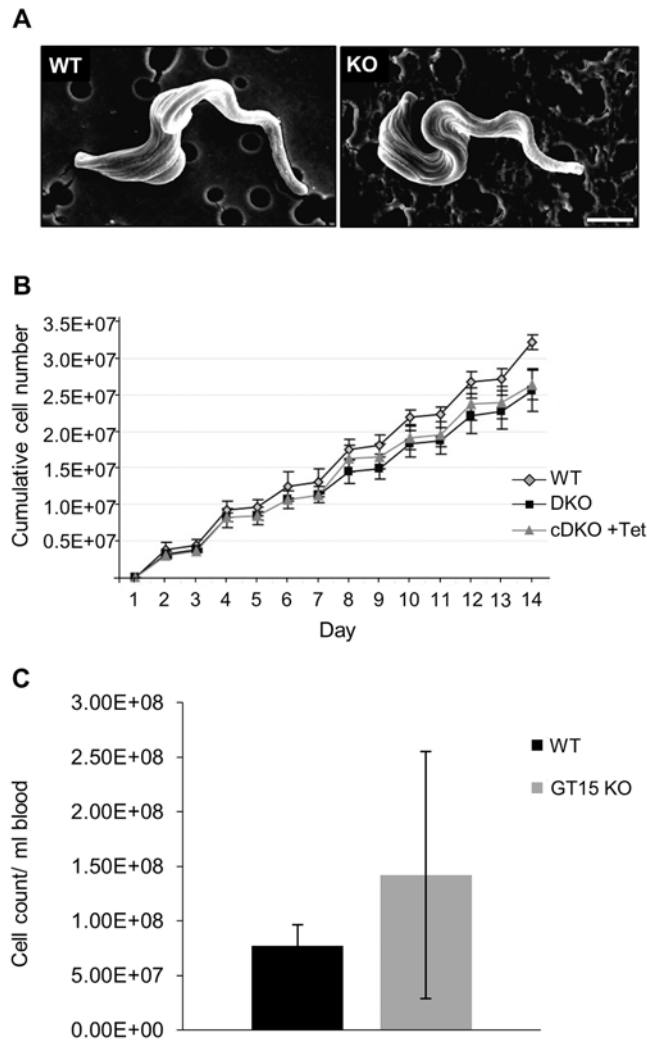


Figure 3

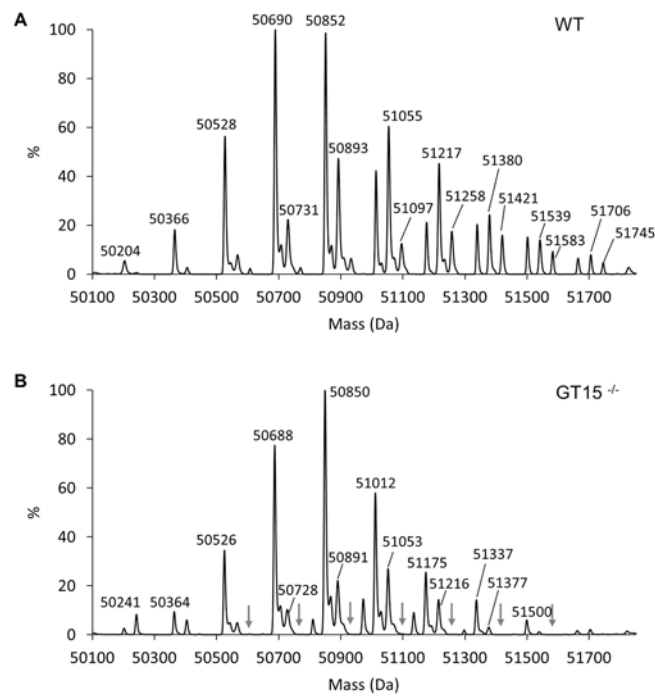


Figure 4

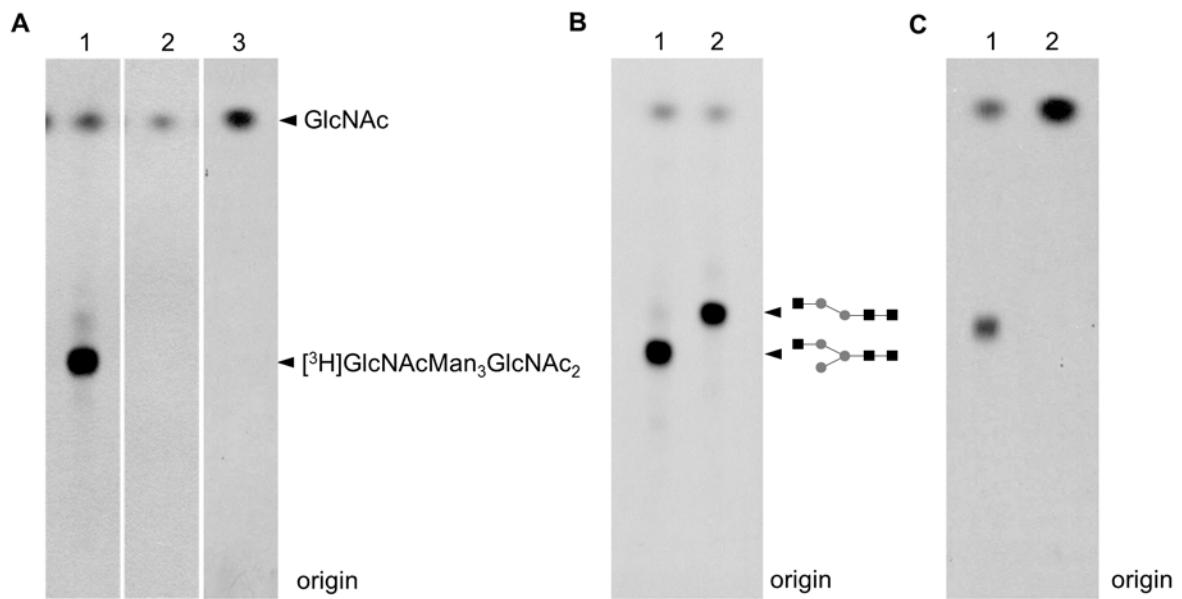


Figure 5

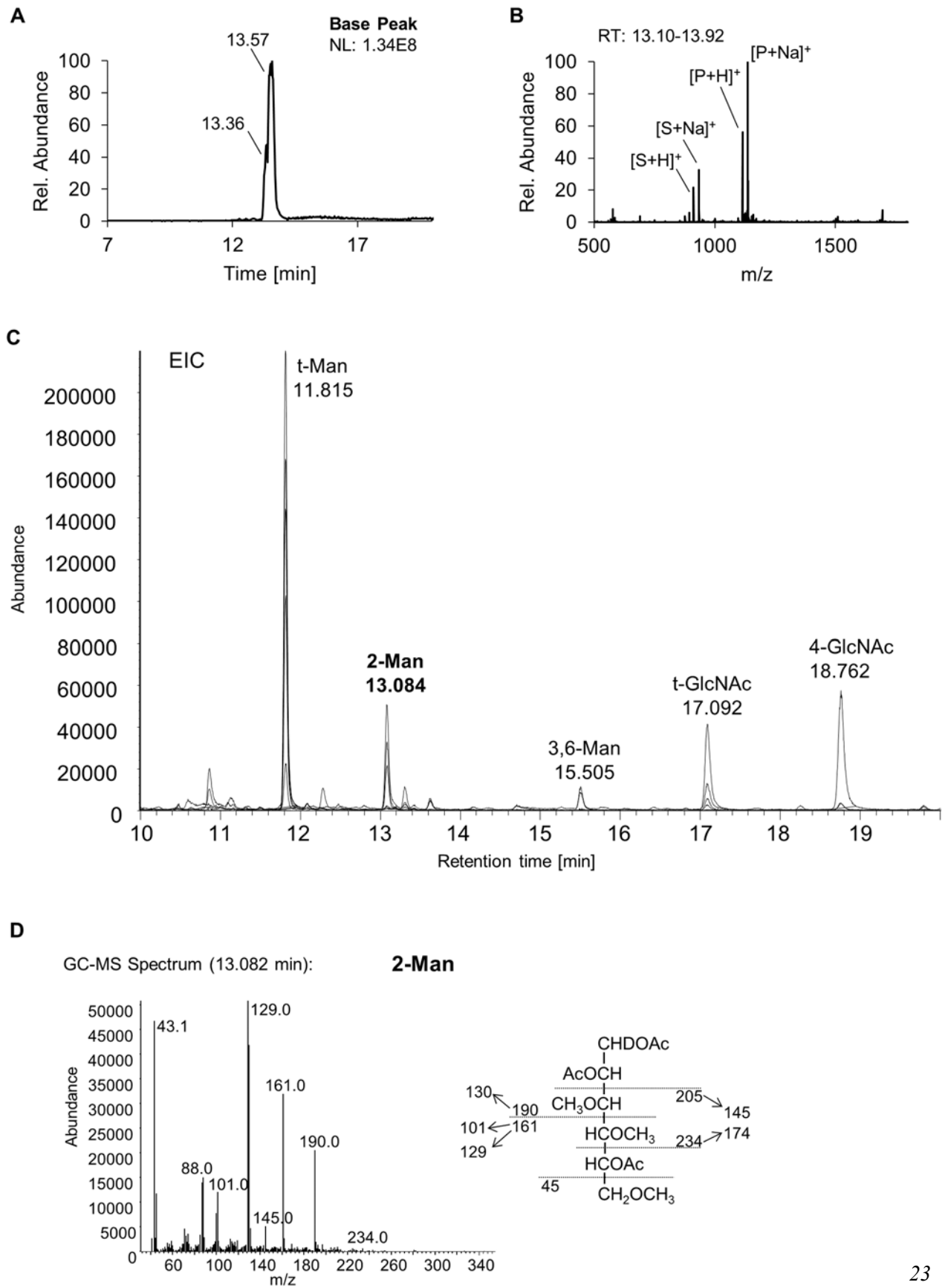


Figure 6

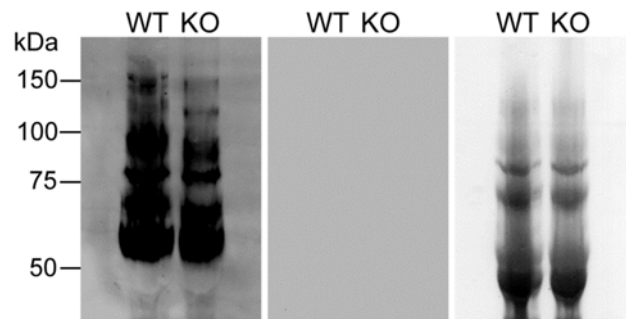


Figure 7

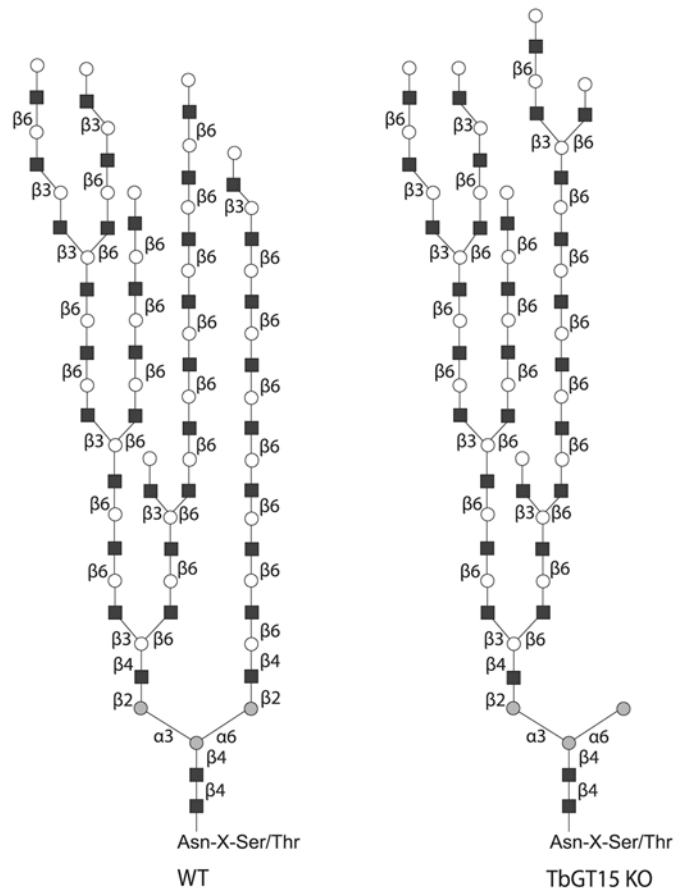
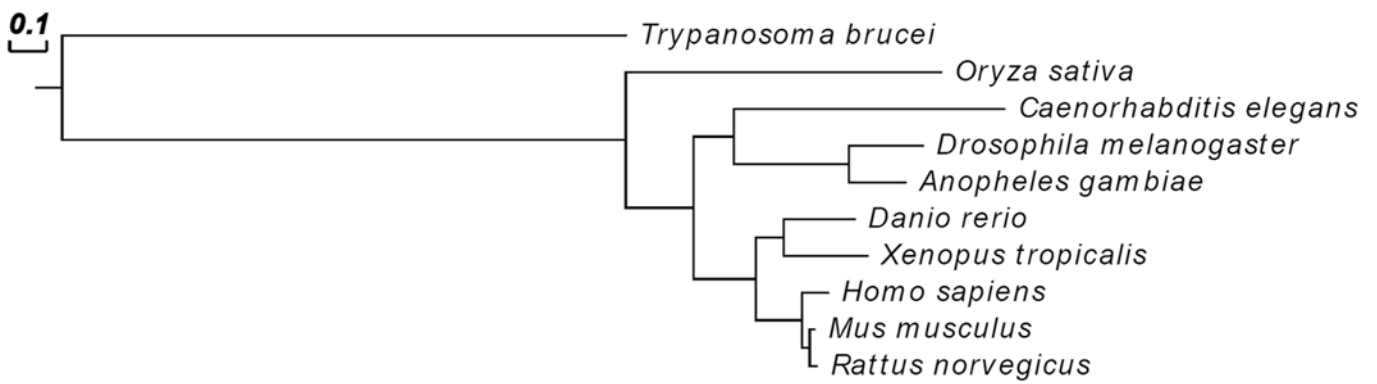


Figure 8



**A gene of the β 3-glycosyltransferase family encodes N-acetylglucosaminyltransferase
II function in *Trypanosoma brucei***

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