DELETION OF THE GLUCOSIDASE II GENE IN TRYpanosoma BrUCeI REVEALS NOVEL N-GLYCOSYLATION MECHANISMS IN THE BIOSYNTHESIS OF VARIANT SURFACE GLYCOPROTEIN

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Running Title: N-glycosylation in T.brucie

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The trypanosomatids are generally aberrant in their protein N-glycosylation pathways. However, protein N-glycosylation in the African trypanosome Trypanosoma brucei, aetiological agent of human African sleeping sickness, is not well understood. Here, we describe the creation of a bloodstream form T.brucie mutant that is deficient in the endoplasmic reticulum enzyme glucosidase II. Characterisation of the variant surface glycoprotein, the main glycoprotein synthesised by the parasite with two N-glycosylation sites, revealed unexpected changes in the N-glycosylation of this molecule. Structural characterisation by mass spectrometry, nuclear magnetic resonance spectroscopy and chemical and enzymatic treatments, revealed that one of the two glycosylation sites was occupied by conventional oligomannose structures while the other accumulated unusual structures in the form of Glcα1-3Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNacβ1-4GlcNac, Glcα1-3Manα1-2Manα1-2Manα1-3(GlcNacβ1-4GlcNcβ1-2Manα1-6)Manβ1-4GlcNacβ1-4GlcNac. The possibility that these structures might arise from Glc3Man9GlcNac9, by unusually rapid α-mannosidase processing was ruled out using a cocktail of α-mannosidase inhibitors. The results suggest that bloodstream form T.brucie can transfer both Man9GlcNac2 and Man9GlcNac3 to the variant surface glycoprotein in a site-specific manner and that, unlike organisms that transfer exclusively GlcMan9GlcNac2, the T.brucie UDP-Glc:glycoprotein glucosyltransferase and glucosidase II enzymes can use Man9GlcNac2 and GlcMan9GlcNac2, respectively, as their substrates. The ability to transfer Man9GlcNac2 structures to N-glycosylation sites destined to become Man9GlcNac2 or complex structures may have evolved as a mechanism to conserve dolichol-phosphate-mannose donors for GPI anchor biosynthesis and points to fundamental differences in the specificities of host and parasite glycosyltransferases that initiate the synthesis of complex N-glycans.

During the bloodstream stage of the life cycle, the cells are covered in a densely packed coat of variant surface glycoprotein (VSG)1. The VSG coat serves as a physical barrier to components of the host complement system and undergoes antigenic variation (1). There are many VSG genes, and each encodes a GPI anchored glycoprotein with one to three N-glycosylation sites (2, 3). The cell line used in this study expresses VSG variant 221 (also known as MiTat1.2). VSG221 carries two occupied N-glycosylation sites, the glycan structures at which have been fully characterised (4). The Asn428 site, five residues from the GPI attachment site, is occupied mostly by oligomannose structures (Manα2GlcNac3), whilst the Asn263 site is occupied by small biantennary structures ranging from Man3GlcNac to Galα1-3Galα1-4GlcNac, biantennary structures

The parasitic protozoan Trypanosoma brucei, transmitted by the tsetse fly, is the causative agent of nagana in cattle and African trypanosomiasis or sleeping sickness in humans.
that are not fully folded (12). Consistent with a role in folding for the monoglucosylated oligosaccharide structures, UGGT shows substrate preference for Man<sub>5</sub>GlcNAc<sub>2</sub>-containing glycans attached to denatured proteins <i>in vitro</i> (13).

The seminal work of Parodi and colleagues has shown that protein N-glycosylation in several trypanosomatid parasites is aberrant, reviewed in (14). None of these organisms can make Dol-P-Glc and so fail to make glucosylated Dol-PP-oligosaccharide precursors. The mature Dol-PP-oligosaccharide species used for transfer to protein vary according to trypanosomatid species. For example, <i>Trypanosoma conhorinii</i>, <i>T.dionisi</i>, <i>Leptomonas samueli</i>, <i>Herpetomonas samuellpesoai</i>, and <i>H.muscarum</i> utilise triantennary Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol; <i>Criithidia fasciculata</i>, <i>Charmosa</i> and <i>Leishmania enriettii</i> utilise biantennary Man<sub>4</sub>GalNAc<sub>2</sub>-PP-Dol; <i>Leishmania mexicana</i>, <i>Leishmania adleri</i> and <i>Blastocrithidia culicis</i> utilise biantennary Man<sub>4</sub>GlcNAc<sub>2</sub>-PP-Dol (15-18). <i>Trypanosoma cruzi</i>, the causative agent of Chagas’ disease in the Americas, utilises Man<sub>4</sub>GlcNAc<sub>2</sub>-PP-Dol during most of its life-cycle but uses both Man<sub>4</sub>GlcNAc<sub>2</sub>-PP-Dol and Man<sub>4</sub>GlcNAc<sub>2</sub>-PP-Dol in its bloodstream trypomastigote stage (19).

The insect-dwelling procyclic form of the African trypanosome <i>T.brucei</i> makes and transfers Man<sub>4</sub>GlcNAc<sub>2</sub>-PP-Dol (20). Although bloodstream form <i>T.brucei</i> also makes Man<sub>4</sub>GlcNAc<sub>2</sub>-PP-Dol (21, 22), it was demonstrated in pulse-chase studies by Bangs <i>et al</i> that one of the two N-glycosylation sites of VSG variant ILTat1.3 receives an Endo H-resistant glycan in the ER (23). This observation, together with the identification by Zamze <i>et al</i> (4) of unusually small (Man<sub>5</sub>GlcNAc<sub>2</sub>) N-glycans on mature VSG221 and the accumulation of substantial amounts of Man<sub>4</sub>GlcNAc<sub>2</sub>-PP-Dol in bloodstream form <i>T.brucei</i> (24), suggests two possible models of protein N-glycosylation in the ER of bloodstream form <i>T.brucei</i> (23): (a) The transfer of Man<sub>5</sub>GlcNAc<sub>2</sub> to some N-glycosylation sites and the transfer of (Endo H-resistant) Man<sub>4</sub>GlcNAc<sub>2</sub> to others. (b) The transfer of Man<sub>5</sub>GlcNAc<sub>2</sub> to both sites and the unusually rapid (immediate) processing of some sites to Endo H-resistant structures in the ER.

In order to help discriminate between these models and to gain further insights into protein N-glycosylation in this parasite, we constructed a bloodstream form <i>T.brucei</i> glucosidaseII null mutant, and used α-mannosidase inhibitors, and assessed their effects on VSG N-glycosylation patterns.

**EXPERIMENTAL PROCEDURES**

**Cultivation of trypanosomes** – Bloodstream form <i>T.brucei</i> genetically modified to express T7 polymerase and the tetracycline represor protein were cultivated in HML-9 medium containing 2.5 µg/ml G418 at 37°C in a 5% CO₂ incubator as described in (25). In some experiments, the parasites were grown for 48 h in the presence of 6 mM 1-deoxynojirimycin (Sigma), the α-glucosidase inhibitor, or with a cocktail of α-mannosidase inhibitors (186 µM kifunensin (Industrial Research Ltd., New Zealand), 100 µM swainsonine and 0.8 mM 1-deoxy-mannojirimycin (Toronto Research Chemicals, Canada). 

**Radioiodelling of trypanosomes** and <i>endo</i>glycosidase digestions - Cells were washed and resuspended at 2.5 x 10<sup>7</sup> /ml in methionine-free DMEM medium, preincubated with or without 0.8 µg/ml tunicamycin (Calbiochem) for 15 min or with and without the aforementioned cocktail of α-mannosidase inhibitors for 30 min and pulse-labelled with 75 mCi/ml [³⁵S]-methionine (Amersham) for 3 min at 37°C. Cells were cooled by addition of 20 ml ice-cold trypanosome dilution buffer (26) containing 1 mM methionine and harvested by centrifugation 800 x g for 10 min at 2°C. The cell pellet was resuspended in 100 µl 0.5% SDS, 20 mM Tris-HCl pH 7.4 containing 1 mM phenylmethylsulfonylfluoride, 0.1 mM tosyl-lysyl-chloromethylketone, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 0.1 M dithiothreitol and boiled for 10 min. Aliquots (5 µl) were combined with 20 µl 50 mM sodium citrate pH 5.5 containing 0.005 Units of Endo H (Roche) or with 20 µl sodium phosphate buffer pH 7.5, 1% NP-40 containing 500 Units PNase F (New England Biolabs). Mock digestion controls were performed with buffer without enzyme. After 3 h at 37°C, samples were mixed with an equal volume of 2 x concentrated SDS sample buffer, boiled and subjected to SDS-PAGE on a 10% gel. The gel was soaked in En³Hance (PerkinElmer) and exposed to Kodak XAR-5 film at -70°C with a Dupont Cronex intensifying screen.

DNA isolation and manipulation - Plasmid DNA was purified from <i>E.coli</i> (DH5α) using Qiagen Miniprep or Maxiprep kits, as appropriate. Gel extraction and reaction clean-up was performed using Qiaquick kits. Custom oligonucleotides were obtained from Thermo Hybaid or the Dundee University oligonucleotide facility. <i>T. brucei</i> genomic DNA was isolated from approximately 2 x 10<sup>8</sup> bloodstream form cells using DNAzol (Helena Biosciences), or from 5 x 10<sup>7</sup> procyclic cells using standard methods.

**Cloning and sequencing of the TbGlcaseII ORF** - A 2517 bp region, including the 2421 bp ORF identified from the <i>T.brucei</i> genome database, was amplified from genomic DNA by PCR using <i>Pfu</i> DNA polymerase with 5'-tattgtttgtggtagttgg-3' and 5'-atgacactgaatcagcc-3' as forward and reverse primers, respectively. The cycling parameters used were 95°C for five minutes, 35 cycles of 95°C for 45 s, 58°C for 45 s and 72°C for 4 min followed by a final 10 min extension time at 72°C. The products of three separate PCRs were ligated into the pCR-Blunt II-TOPO vector (Invitrogen) and one representative clone from each was used for triple pass DNA sequencing.

**Generation of gene replacement constructs** - The 500-bp 5'- and 522-bp 3'-UTR sequences immediately adjacent to the start and stop codons of the <i>TbGlcaseIIa</i> ORF were PCR amplified from genomic DNA using <i>Taq</i> with 5'-tcaagcttCAGGCGCCCGcatgctggagc-3' and 5'-ttggcgttattaactgaactgactgcgttgttctactc-3' as forward and reverse primers, respectively. The two PCR products were used together in a further PCR reaction to yield a product containing the 5'-UTR linked to the 3'-UTR by a short HindIII, PmeI and BamHI cloning site (underlined) and NotI restriction sites at each end (capital letters). The product was cloned into the pCR4-TOPO vector (Invitrogen) by topoisomerase mediated ligation. Antibiotic resistance markers were cloned into the HindIII / BamHI restriction sites between the two UTRs to produce two constructs, one containing the puromycin acetyltransferase (PAC) drug resistance gene and one containing the
Transformation of bloodstream form T. brucei - Constructs for gene replacement and ectopic expression were purified using the Qiagen Maxiprep kit, digested with NotI to linearise, precipitated and washed twice with 70% ethanol and redissolved in sterile water. The linearised DNA was electroporated into T. brucei bloodstream cells (strain 427, variant 221) that were stably transformed to express T7 RNA polymerase and the tetracycline repressor protein under G418 selection (25). Cell culture and transformation was carried out as previously described (25, 27).

Southern blotting - Aliquots of genomic DNA isolated from 100 ml bloodstream form T. brucei cultures (approximately 2 x 10^8 cells) were digested with various restriction enzymes. Fluoroscein labelled probes were generated using the CDP-star random prime labelling kit (Gene Images); 250 ng template was used in a reaction volume of 50 µl and incubated for 90 min. Aliquots of 8 µl were used for each Southern blot experiment.

Small scale VSG isolation - Soluble form VSG was isolated from 100 ml cultures, containing approximately 2 x 10^8 bloodstream form T. brucei. The cultures were chilled on ice-water and centrifuged at 2500 g for 10 min. The pellet was washed twice in trypanosome dilution buffer (26) and transferred to a 1.5 ml Eppendorf tube. The pellet was resuspended in 300 µl lysis buffer (10 mM NaH2PO4 buffer pH 8.0, containing 0.1 mM TLCK, 1 µg / ml leupeptin and 1 µg / ml aprotinin) prewarmed to 37°C and incubated for 5 min at the same temperature. The sample was centrifuged at 14,000g for 5 min and the supernatant applied to a 200 µl DE52 anion exchange column pre-equilibrated in lysis buffer. Fresh lysis buffer (800 µl without protease inhibitors) was applied in four stages and the pooled column eluate concentrated and dialyzed with water on a YM-10 spin concentrator (Microcon). The final sample of 50-100 µg sVSG221 was recovered in a volume of 100 µl water.

ES-MS analysis of intact VSG - Samples of the small-scale VSG preparations were diluted to approximately 0.05 µg/µl in 50% acetonitrile, 1% formic acid or 50% methanol, 1% formic acid, loaded into nanotips (Micromass type F) and declustering potentials of 900 V and 36.0 min. Aliquots of 50 µl, 1% formic acid and aliquots were loaded into nanotips (Micromass type F) for mass spectrometry. Pronase glycopeptide fractions were analysed by positive ion ES-MS/MS in neutral loss scanning mode (neutral loss of m/z 81 for the loss of terminal hexose from doubly-charged parent ions) using a Micromass Ultima triple quadrupole mass spectrometer. Tip and cone voltages were 1 kV and 40 V and the collision energy was 15 V with argon as the collision gas at 3 x 10^4 Torr. Glycopeptide ions identified in this experiment were analysed on a QToF2 instrument (using the same source conditions) or on a Q-StarXL instrument, with tip and declustering potentials of 900 V and 60V, in product ion scanning mode using collision energies of 30-60 V.

Large scale VSG and VSG N-glycan isolation - TbGlcaseIα- null mutant parasites (2 x 10^11) were harvested from the infected blood of 20 rats as previously described (27). The parasites were lysed by osmotic shock and the sVSG released into the supernatant purified using DE-52 anion exchange chromatography according to the method of Cross (28). The VSG was further purified by gel filtration on a 850 x 2.5 cm Sephacryl S200 column eluted with 100 mM ammonium bicarbonate at 10 ml/h (29). The protein peak fractions were pooled to yield 36 mg sVSG following dialysis against water and lyophilisation. A sample of sVSG221 (25 mg) was incubated in 200 µl 0.5% SDS, 1% β-mercaptoethanol (100°C, 30 min), cooled and diluted with 800 µl 125 mM sodium phosphate buffer (pH 7.0), 0.625% Triton-X100, 25 mM EDTA, 1% β-mercaptoethanol. The denatured glycoprotein was then treated with 50 U PNGase F (Roche) for 16 h at 37°C. The PNGase-F digest was made 75% with respect to ice-cold ethanol and incubated at -20°C overnight to precipitate the bulk of the protein. After centrifugation, the supernatant was dried and dissolved in 100 mM ammonium bicarbonate for gel filtration on a Sephacryl S200 column (13 x 1.5 cm) eluted at 10 µl/h. Fractions of 1 ml were collected and 10 µl aliquots spotted onto a silica gel-60 TLC plate (Merck) and stained for carbohydrate with orcinol reagent. The carbohydrate-positive fractions, containing the released N-glycans, were recovered close to the included volume, well separated from a major A280 absorption peak containing protein and detergent micelles. The glycan-containing fractions were pooled, lyophilised and desalted by passage through 0.2 ml Chelex 100 (Na+) over 0.5 ml Dowex AG50 (H+) over 0.5 ml Dowex AG3 (OH-) over 0.2 ml QAE-Sephadex A25 (OH-). The eluate and four column washings with 1.4 ml water were pooled, dried by rotary evaporation and dissolved in a 100 µl water.

N-glycan fractionation - The N-glycan pool released from sVSG221 was separated by Dionex HPAC on a 4.6 x 250 mm Carbopack PA1 column eluted at 0.6 ml/min with 5 mM sodium acetate in 0.1 M NaOH for 5 min followed by a linear gradient to 150 mM sodium acetate in 0.1 M NaOH over 55 min. Sodium ions were removed from the eluate on-line with a Dionex ARRS unit. Aliquots (1%) of each 0.3 ml fraction were applied to a silicagel-60 TLC plate and stained with orcinol. Carbohydrate-positive fractions were identified eluting between 30.5 and 36.0 min. Aliquots of these fractions (3 µl) were mixed with an equal volume of acetonitrile containing 2% formic acid and analysed by positive ion nanospray ES-MS to detect [M+2H]^2+, [M+H+Na]^2+ and [M+2Na]^2+ ions of N-glycans. Fractions were pooled according to the ES-MS data. Fraction pools A

hygromycin phosphotransferase (HPT) hygromycin drug resistance gene.

Hygromycin B + D (strain 427, rocon). The final sample of 50 cm Sephacryl S200 column eluted through potentials of 900 V and 36.0 min. Aliquots of 50 µl, 1% formic acid and aliquots were loaded into nanotips (Micromass type F) for mass spectrometry. Pronase glycopeptide fractions were analysed by positive ion ES-MS/MS in neutral loss scanning mode (neutral loss of m/z 81 for the loss of terminal hexose from doubly-charged parent ions) using a Micromass Ultima triple quadrupole mass spectrometer. Tip and cone voltages were 1 kV and 40 V and the collision energy was 15 V with argon as the collision gas at 3 x 10^4 Torr. Glycopeptide ions identified in this experiment were analysed on a QToF2 instrument (using the same source conditions) or on a Q-StarXL instrument, with tip and declustering potentials of 900 V and 60V, in product ion scanning mode using collision energies of 30-60 V.

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%E contained predominantly the following structures: (A) HexαHexNAc2, (B) HexαHexNAc2, (C)HexαHexNAc2, (D)HexαHexNAc2, and (E) HexαHexNAc2. Each fraction was analysed by GC-MS monosaccharide composition analysis (30) to estimate yield.  

\[ ^1H-NMR \] One-dimensional 500 MHz \[ ^1H-NMR \] spectra of fractions B and C were obtained using the zgpr pulse program on a Bruker AM500 spectrometer equipped with a 5 mm triple resonance cryoprobe. Samples were repeatedly exchanged into \[ ^2H_2O \], dissolved in 350 \( \mu l \) \[ ^2H_2O \] and transferred to a Shigemi tube. Experiments were performed at 300K, with a sweep width of 10 ppm; 256 scans were recorded after 8 dummy scans. Two-dimensional experiments were performed under the same conditions. COSY and ROESY experiments were performed with Watergate suppression and TOCSY spectroscopy used a phase-sensitive mlev program.  

Permethylation and ES-MS/MS of N-glycans - Samples recovered from NMR were exchanged back into \[ ^2H_2O \] by passage through a short column (20 x 1 cm) of BioGel P2 (BioRad) equilibrated and eluted with water. Samples were dried and permethylated by the sodium hydroxide method, as described in (30). The permethylated glycans were dissolved in 100 \( \mu l \) 80% acetonitrile and aliquots (2 \( \mu l \)) were mixed with an equal volume of 80% acetonitrile, 1 mM sodium acetate prior to loading into nanotips (Micromass type F) for positive ion ES-MS and ES-MS/MS on a QToF mass spectrometer (Micromass, U.K.). Tip and cone voltages were 1 kV and 40 V, respectively, and the collision energy was 45-70 V.  

Methylation linkage analysis by GC-MS - The remainder of the permethylated glycan samples were subjected to acid hydrolysis, NaB\[\text{H}_4\]-reduction and acetylation (to yield partially methylated alditol acetates, PMAAs) and analysed by GC-MS as described in (30). The PMAAs were analysed on an Agilent HP-5 column and on a Supelco SP2380 column (the latter to allow resolution of the non-reducing terminal-Man and non-reducing terminal-Glc PMAAs).  

Tritium labelling and HPTLC analysis of glycans - Samples of pools B, C and D (2 mmol each) were dried and reduced with 5 \( \mu l \) 5.5 mM NaB\[\text{H}_4\] in 16.6 mM NaOH (16 h, 22°C). Excess reductant was added (5 \( \mu l \) 0.5 M NaB\[\text{H}_4\]) and the incubation continued for 3 h. The samples were acidified with 10 \( \mu l \) 1 M acetic acid in a fume hood, passed through 0.2 ml Dowex AG50 (H\(^+\)) and dried. Boric acid was removed by co-evaporation with 2 x 250 \( \mu l \) 5% acetic acid in methanol and 2 x 250 \( \mu l \) methanol. The labelled glycans were dissolved in water and applied to 3 cm-wide strips of Whatman 3MM paper for downward paper chromatography in butan-1-ol, ethanol, water (4:1:0.8). Labelled glycans were recovered from the origin by elution with water and desalted by passage through a column of 100 \( \mu l \) each Chelex 100 (Na\(^+\)) over Dowex AG50 (H\(^+\)) over Dowex AG3 (OH\(^-\)) over QAE-Sephadex A25 (OH\(^-\)). The column was eluted four times with 400 \( \mu l \) water and the combined eluates were dried by rotary evaporation. The \( ^3\)H-labelled glycans were then purified by Dionex HPACE, as described above.  

The same strategy was used to generate highly purified labelled standards of Man\(\alpha\)GlcNAc\(\beta\) (i.e., Man\(\alpha\)-1-6[Man\(\alpha\)-1-3]Man\(\alpha\)-1-6[Man\(\alpha\)-1-2Man\(\alpha\)-1-3]Man\(\beta\)-1-4GlcNAc\(\beta\)-1-4[1-\( ^3\)H]GlcNAc-ol and NA2 (i.e., Gal\(\beta\)-1-4GlcNAc\(\beta\)-1-2Man\(\alpha\)-1-3)Man\(\beta\)-1-4GlcNAc\(\beta\)-1-4[1-\( ^3\)H]GlcNAc-ol. The unlabelled Man\(\alpha\)GlcNAc\(\beta\) and NA2 glycans were obtained from (Dextra Labs, U.K.). The Thy-1 GPI standard (i.e., Man\(\alpha\)-1-2Man\(\alpha\)-1-6[Gal\(\beta\)NaAc\(\beta\)-1-4]Man\(\beta\)-1-4[1-\( ^3\)H]2,5-anhydromannitol) was prepared from rat brain Thy1 glycoprotein, as described in (31).  

Aliquots equivalent to 10,000 cpm (before and after digestion with jack bean \( \alpha \)-mannosidase (Calbiochem) and \( E.coli \) Bl-4 galactosidase (Roche) or partial acetylation (32)) were applied to a 10 cm aluminium backed silicagel-60 HPTLC plate (Merck) and developed with butan-1-ol : ethanol : water (4 : 3 : 3 v/v/v) three times (32). Radio-labelled components were detected by fluorography at -70°C after spraying with En\(\alpha\)Hance (PerkinElmer) using Kodak X-OMAT AR film and an intensifying screen.  

RESULTS

Endo H resistant N-glycans are added to one glycosylation site of VSG221 – Variant 221 trypanosomes were pulse labelled in culture with \( ^{[35S]}\) methionine for 3 min and the cell lysates were analysed by SDS-PAGE and fluorography before and after PNGase F and Endo H treatment. The results show that two forms of VSG are apparent after 3 min pulse labelling (Fig. 1, lanes 1 and 3); a weakly labelled 53 kDa unglycosylated band (that co-migrates with VSG synthesised in the presence of the \( N\)-glycosylation inhibitor tunicamycin, data not shown) and a strongly labelled upper 57 kDa band that co-migrates with mature VSG221 containing 2 occupied N-glycosylation sites at Asn263 and Asn428 (4). Treatment with PNGase F, an endoglycosidase that removes all types of N-glycan, converted the upper band to the lower band, as expected (Fig. 1, lanes 4). However, treatment with Endo H, an endoglycosidase that removes only conventional triantennary oligomannose and hybrid N-glycans, converted the upper band to an intermediate 54.5 kDa band representing VSG bearing a single Endo H-resistant N-glycan (Fig. 1, lane 2).

These results show that newly synthesised (ER-resident) VSG221 contains one conventional Endo H-sensitive N-glycan and one Endo H-resistant N-glycan. This reproduces the observation made for a similar doubly-N-glycosylated VSG (variant 1Ltax1.3) (23) and shows that bloodstream form \( T.brueci \) cells exhibit both a conventional and aberrant features of protein N-glycosylation. Thus, whereas in other eukaryotes all N-glycans are Endo H sensitive until glycoproteins are processed in the Golgi apparatus (several minutes after synthesis in the ER), in \( T.brueci \) some N-glycans are Endo H resistant upon or immediately after transfer to VSG polypeptide in the ER.

Bioinformatic analysis - Local alignment searching (tBLASTn) (33) of \( T.brueci \) genome data held in GeneDB at The Sanger Institute with yeast and/or mouse genes associated with the biosynthesis of Dol-PP-oligosaccharide donors, their transfer to Asn-X-Ser/Thr sequons and subsequent ER processing was performed (Table I). The search using the mouse glucosidase II alpha subunit as a query sequence identified two putative open reading frames showing significant sequence similarity (62% and 51%). The homologue with the greatest similarity to the query (gene number Tb10.05.0080) was chosen for further analysis and was later demonstrated to be the \( T.brueci \)
glucosidase II alpha subunit (*TbGlcaseIIα*). The second homologue (gene number *Tb*11.01.2140) showed greater similarity to human lysosomal alpha glucosidase and was not studied further. A putative homologue of the human glucosidase II β-subunit was also found (Table I), suggesting that the *T. brucei* contains a conventional α/β glucosidase II assembly in the ER, although the *T. brucei* β-subunit homologue lacks an identifiable ER retention sequence. Homologues of UDP-Glc:glycoprotein glucosyltransferase (UGGT) and calreticulin (CRT) were also found (Table I). However, like in *Trypanosoma cruzi* (34), no calnexin (CNX) homologue was apparent, these data suggest that *T. brucei* has a relatively conventional eukaryotic calreticulin-mediated glycoprotein refolding quality control system (7, 10, 11). On the other hand, BLAST searches for homologues of eukaryotic genes involved in the biosynthesis of the conventional GlcManGlcNAc2-PP-Dol N-glycan precursor indicated that the ALG6, 8 and 10 genes (encoding the three Dol-P-Glc-dependant α-glucosyltransferases) were absent as were obvious homologues for Dol-P-Glc synthetase (ALG5) and glucosidase II (Table I). Similar conclusions were reported recently by Samuelson et al (35).

**Cloning and Sequencing of the *TbGlcaseIIα* ORF** - The *TbGlcaseIIα* ORF was amplified from *T. brucei* genomic DNA by PCR using *Pfu* polymerase. Three clones from separate amplifications were sequenced three times each. The consensus sequence (accession number AJ865333) revealed eleven base differences compared to the genome database assembly (gene number *Tb*10.05.0080). All of the differences were silent with respect to predicted amino acid sequence and probably represent differences between the strain used for the genome sequencing project (TREU927/4) and that used in this study (strain 427). The amino acid sequence suggests that *TbGlcaseIIα* encodes a glycosyl hydrolase family 31 protein with a cleavable N-terminal signal peptide for import into the ER (residues 1-19) and two potential N-glycosylation sites at Asn122 and Asn736.

**Generation of *TbGlcaseIIα* null and conditional null mutant cell lines** - Strain 427 bloodstream form *T. brucei* parasites expressing VSG221 and transformed to stably express T7 polymerase and the tetracycline repressor protein under G418 expression vector and the tetracycline repressor protein under G418 selection, was replaced by homologous recombination (ATbGlcaseIIα::HPT) were selected with hygromycin. The second *TbGlcaseII* allele was replaced in a ATbGlcaseIIα::HPT clone in the same way with puromycin acetyltransferase (PAC). Hygromycin and puromycin resistant clones (ATbGlcaseIIα::HPT / ATbGlcaseIIα::PAC null mutants) were selected. Southern blot analysis of the wild-type, ATbGlcaseIIα::HPT and ATbGlcaseIIα::HPT / ATbGlcaseIIα::PAC null mutant cells with a probe to the *TbGlcaseIIα* 5'-UTR confirmed that both alleles had been replaced (Fig. 2B). The null mutant cell line had no noticeable differences in gross morphology. However, it exhibited marginally slower growth in culture (doubling time 6.4±0.5 h) than the parental cell line (doubling time 5.5±0.5 h).

A tetracycline-inducible ectopic copy of the *TbGlcaseIIα* gene was introduced into a null mutant clone using the pLe882 vector (25) and phleomycin selection to yield ΔTbGlcaseIIα5 / ΔTbGlcaseIIα::HPT / ΔTbGlcaseIIα::PAC conditional null mutant clones.

**Characterisation of intact VSG from wild type and *TbGlcaseIIα* null and conditional null mutant cell lines** - The cell lines were grown in vitro and samples of approximately 2 x 10^8 cells from 100 ml of culture were processed to yield 50-100 µg of purified sVSG221. Aliquots were analysed by positive ion ES-MS. The deconvoluted mass spectra of the intact glycoproteins are shown in (Fig 3). The wild type profile (Fig. 3A) shows the range of different glycoforms that arise from known heterogeneity in the GPI anchor (36) and N-glycan sites (4) (Table II). VSG from the *TbGlcaseIIα* null mutant showed a significant difference in glycoform pattern with a shift of glycoforms to higher mass equivalent to approximately three hexose units (486 Da) (Fig. 3B). This change in glycoform pattern was reversed in the tetracycline-induced conditional null ('add-back') mutant (Fig. 3C), demonstrating that the change in VSG glycoform profile was a direct result of *TbGlcaseIIα* gene deletion. An identical change in VSG glycoforms to that seen in the null mutant was observed when wild type cells were grown for 48 h in the presence of 6 mM 1-deoxynojirimycin, a broad specificity α-glucosidase inhibitor (37), prior to VSG isolation (Fig. 3D). The latter result suggests that the *TbGlcaseIIα* gene analysed in this study is the only ER α-glucosidase involved in N-glycan processing in *T. brucei*.

**Characterisation of VSG Pronase glycopeptides from wild type and *TbGlcaseIIα* null mutant cell lines** - To probe the nature of the changes in VSG glycosylation induced by deletion of the *TbGlcaseIIα* gene, aliquots of wild-type and null mutant VSG samples (approximately 50 µg) were digested with Pronase and analysed by ES-MS/MS in neutral loss scanning mode. This method filters the spectrum for doubly-charged glycopeptide ions that can lose a terminal hexose residue (m/z 81) when they undergo collision induced dissociation (Fig. 4A and B). These data showed no apparent changes in the masses of the GPI-peptide fragments or the C-terminal (Asn428) Hexa-, HexNAc-, containing glycopeptides. The identities of these ions were confirmed by daughter ion ES-MS/MS on a QToF2 mass spectrometer; eg. (Fig. 4C and D).

The absence of any hint of Hexa,GlcNAc2,NTT ions in (Fig. 4B) is noteworthy. This suggests that deletion of the *TbGlcaseIIα* gene does not lead to any GlcMan,GlcNAc structures attached to the C-terminal Asn486 site. On the other hand, the internal (Asn263) glycopeptides were changed from the previously characterised Hexa,HexNAc2, Hexa,HexHAC2, and Hexa,HexNAc2-containing species (4) to Hexa,HexNAc2-containing species (Fig 4B). The identities of these ions were confirmed by daughter ion ES-MS/MS on a QToF2 mass spectrometer; eg. (Fig. 4E and F). In order to characterise these changes, N-linked glycans were isolated from the null mutant VSG for detailed structural analysis.
Structural characterisation of the N-linked glycans of sVSG221 isolated from TbGlcaseIIΔα null mutant cells – A large-scale VSG221 preparation was made from TbGlcaseIIΔα null mutant cells. The N-linked glycans were released from 25 mg sVSG221 using PNGase F and separated by high-pH anion exchange chromatography (HPAEC). Analysis of the column fractions by ES-MS showed that they eluted between 30.5 and 36.0 min in the order Hex3HexNAc2, HexHexNAc2, HexHexNAc2, HexHexNAc2 and HexHexNAc2. The HexHexNAc2 and HexHexNAc2 species from the Asn428 glycosylation site (see above) were studied by positive ion ES-MS/MS after permethylation. The daughter ion spectra of the [M+2Na]+ pseudomolecular ions were identical to those of conventional Man,GlcnAc2 and Man,GlcnAc2 oligomannose glycans (data not shown) and were in agreement with the structures observed previously by Zamze et al (4). Notably, there was no evidence for the presence of a terminal glucose residue in these structures (that would otherwise give rise to a B-type daughter ion at m/z 853 corresponding to a linear Hex4 branch; see below). To confirm this, aliquots of the native glycans were radiolabelled by reduction in NaB3H4 and analysed along with radiolabelled oligomannose standards by HPTLC with and without treatment with jack bean α-mannosidase (JBAM). Both structures were fully digested to Manβ1-4GlcnAcβ1-4GlcnAc-Ol, confirming the lack of any JBAM-resistant αGlc residues in the parent structures (data not shown).

The novel Hex3HexNAc2 structure from the Asn263 site was analysed by GC-MS compositional analysis, 1-D and 2-D 1H-NMR and, following permethylation, ES-MS/MS and GC-MS methylation linkage analysis.

The 1-D NMR spectrum revealed eight well-resolved anomeric protons. The chemical shift and J1,2 coupling constant values suggested the presence of an αGlc residue, four αMan residues, a βMan residue and two βGlcNAc residues (38) (Fig. 5A). These assignments were confirmed when intra-residue connectivity networks were traced from COSY, ROESY and TOCSY spectra, which allowed assignment of additional protons in each spin-system (Table III). Using the residue descriptors shown in (Fig. 5A), inter-residue connectivities were observed in the ROESY spectrum between G3-H1 and D1-H3, D1-H1 and C-H2, C-H1 and 4-H2 (Fig. 5B). This suggests the linear sequence Glcα1-3Manα1-2Manα1-. These assignments were consistent with the ES-MS/MS data that show a linear Hex branch, indicated by the m/z 853 B-type and m/z 953 Y-type daughter ions (Fig. 6A). The GC-MS methylation linkage analysis showed the presence of a terminal Glc residue together with terminal Man, 3-O-substituted Man, 2-O-substituted Man and 3,6-di-O-substituted Man residues (Table IV). To assess whether the Glcα1-3Manα1-2Manα1-2Man branch was attached to the 3- or the 6-position of the βMan residue of the Manβ1-4GlcnAcβ1-4GlcnAc unit, the NaB3H4-reduced oligosaccharide was analysed by HPTLC before and after partial acetylation. This treatment, which is selective for the cleavage of Manα1-6Man glycosidic bonds, resulted in the loss of one hexose residue (Fig. 7A, lanes 1 and 2), consistent with a single αMan attached to the 6-position and the Glcα1-3Manα1-2Manα1-2Man branch attached to the 3-position (Fig. 7C). Taken together, these data provide support for the structure proposed in (Fig. 5A). This structure contains a conventional biantennary Manα1-3(Manα1-6)Manβ1-4GlcnAcβ1-4GlcnAc core that is extended on the 3-arm as follows: Glcα1-3Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcnAcβ1-4GlcnAc. The likely origin of this structure is discussed later.

Similar analyses of the HexHexNAc2 structure showed that this material had an additional terminal βGlcNAc residue on the 6-arm of the Manα1-3(Manα1-6)Manβ1-4GlcnAcβ1-4GlcnAc core. Thus, the NMR data are similar to those for the HexHexNAc2 except for the presence of an additional βGlcNAc residue, residue 5’ (Fig. 5C) (Table IV). The ES-MS/MS data again show that a linear Hex branch, indicated by the m/z 853 B-type daughter ion, together with a non-reducing terminal HexNAc residue that gives rise to the m/z 282 B-type and m/z 1791 Y-type daughter ions (Fig. 7B). Methylation linkage analysis (Table IV) also identified this non-reducing terminal GlcNAc residue, together with a disappearance of terminal Man and the appearance of additional 2-O-substituted Man. Partial acetylation of the NaB3H4-reduced Hex3HexNAc2 oligosaccharide produced the same HexHexNAc2 product as the NaB3H4-reduced HexHexNAc2 oligosaccharide (Fig. 7A, lanes 3 and 4), supporting the notion that the additional βGlcNAc residue is attached to the 4’ residue (Fig. 7C). Taken together, these data provide support for the structure proposed in (Fig. 5C). This structure contains a conventional biantennary Manα1-3(Manα1-6)Manβ1-4GlcnAcβ1-4GlcnAc core that extended on the 3-arm and the 6-arm as follows: Glcα1-3Manα1-2Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcnAcβ1-4GlcnAc.

There was insufficient HexHexNAc2 material for NMR and methylation linkage analysis. However, the m/z 853 B-type daughter ion in the ES-MS/MS spectrum clearly indicates the presence of the linear Hex branch, together with a terminal HexHexNAc unit (indicated by the intense m/z 486 B-type daughter ion) (Fig. 6C). This new terminal Hex residue was deduced to be a βGal residue in 1-4 linkage to the underlying GlcNAc residue by digestion of the NaB3H4-reduced HexHexNAc2 oligosaccharide with E.coli β1-4-specific β-galactosidase (Fig. 7B, lanes 5 and 6). As expected, neither the HexHexNAc nor the HexHexNAc2 were digested by β-galactosidase (Fig. 7B, lanes 1-4). The location of the Galβ1-4GlcnAc unit was inferred by partial acetylation of the NaB3H4-reduced Hex3HexNAc2 oligosaccharide. Thus, the terminal product of this reaction was also Hex3HexNAc2 (Fig. 7A, lanes 5 and 6), suggesting that it is attached to the 4’ residue (Fig. 7C). These data are consistent with the structure: Glcα1-3Manα1-2Manα1-2Manα1-3Galβ1-4GlcnAcβ1-2Manα1-6Manβ1-4GlcnAcβ1-4GlcnAc.

The effects of α-mannosidase inhibitors on wild-type VSG221 N-glycosylation - The novel structures found at the Asn263 glycosylation site in the glucosidase II null mutant point to the transfer of Man,GlcNAc2, its subsequent glycosylation by UGGT and processing by GlcNAc- and Gal-transferases. However, it is also conceivable that trypanosomes might perform unusually rapid α-mannosidase trimming of a conventional Man,GlcNAc2 structure to Man,GlcNAc2 at that site.

To address this possibility, we repeated the experiment shown in (Fig. 1) after pre-incubation with a cocktail of
cell-permeable α-mannosidase inhibitors (186 μM kifunensin, 100 μM swainsonine and 0.8 mM 1-deoxymannojirimycin). The results (Fig. 8A) are very similar to those in (Fig. 1); i.e., after a 3 min pulse-label one of the 2 N-glycosylation sites is already endo H resistant, suggesting that Asn263 is not rapidly processed by α-mannosidases from endo H-sensitive Man,GlcNAc2 to endo H-resistant Man,GlcNAc3. To confirm this, we grew wild-type trypanosomes for 48 h in the presence and absence of the α-mannosidase inhibitor cocktail and assessed the effects of the inhibitors on VSG221 N-glycosylation by mass spectrometry. Aliquots of purified VSG221 were analysed by positive ion ES-MS and the mass spectra of the intact glycoproteins are shown in (Fig. 8B and C). The wild type profile (Fig. 8B) shows the range of different glycoforms that arise from known heterogeneity in the GPI anchor (37) and N-glycan sites (4) (Table II). VSG from the mannosidase inhibitor-treated cells showed a significant difference in glycoform pattern, with a shift of glycoforms to higher mass (Fig. 8C) and (Table II). These data indicate that the mannosidase inhibitor cocktail is active.

To assess the effects of the mannosidase inhibitors on the individual N-glycosylation sites, aliquots of wild-type and mannosidase inhibitor-treated VSG samples were digested with Pronase and analysed by ES-MS/MS in neutral loss scanning mode, as described earlier. These data showed no apparent changes in the masses of the GPI-peptide fragments but, as expected, a significant reduction in the α-mannosidase-mediated processing of Man,GlcNAc2 to Man,GlcNA3 and Man,GlcNA3, at the C-terminal Asn428 glycosylation site, compare (Fig. 8D) with (Fig. 8E). The glycans at the internal Asn263 site were 2 hexose units larger in the α-mannosidase-treated sample than the wild type-sample, compare (Fig. 8D) with (Fig. 8E). This is consistent with inhibition of the α-mannosidases responsible for trimming the 3-arm of the biantennary Man,GlcNAc2 to Man,GlcNAc3 but inconsistent with the rapid α-mannosidase processing of a conventional Man,GlcNAc2 precursor at this glycosylation site.

Based on the data of Zamze et al (4) and this paper, the differences in VSG221 glycosylation between wild-type, TbgEIIα-null mutant trypanosomes and α-mannosidase inhibitor-treated wild type trypanosomes are summarised in (Fig. 9).

DISCUSSION

It is clear that, despite slightly retarded growth in the null mutant, TbgEIIα is a non-essential enzyme for the growth of bloodstream form T. brucei in vitro and in vivo. We were also able to successfully differentiate the null mutant into procyclic form T. brucei in vitro (data not shown), suggesting that the gene is also not required for differentiation to, or survival of, the procyclic form of the parasite. In this regard, our results are similar to those of Parodi and colleagues, who have shown that, in the related American parasite T. cruzi, other components of the ER glycosylation-dependent quality control system (i.e., calreticulin and UGGT) can be deleted with only moderate effects on parasite growth, differentiation and infectivity (34, 39). However, analysis of the biochemical phenotype of the T. brucei glycosidase II null mutant, together with BLAST searches for glycosylation gene homologues, has helped to reveal and explain some of the idiosyncrasies of protein N-glycosylation in the African trypanosome, summarised in (Fig. 10).

While the T. brucei genome contains homologues of Dol-P-Man synthase and all of the known components needed to synthesise Man,GlcNAc2,PP-Dol, to flip this species into the lumen of the ER and to assemble Man,GlcNAc2,PP-Dol, trypanosome equivalents of the three Dol-P-Glc-dependent α-glucosyltransferases (encoded by ALG6, ALG8 and ALG10 in yeast) and Dol-P-Glc synthase (ALG5) are absent (Table I) and (35). This is in agreement with published data that show that the largest Dol-PP- oligosaccharide species observed in both bloodstream form and procyclic form T. brucei is Man,GlcNAc2,PP-Dol (20-22) and not the Glc₄Man,GlcNAc2,PP-Dol species common to most other eukaryotes (7, 10, 11). Perhaps unsurprisingly, a homologue of glucosidase I (that removes the terminal αGlc residue from Glc₄Man,GlcNAc2-protein) is also absent from the genome. On the other hand, there appears to be components of a calreticulin- (but not claxin- ) mediated glycoprotein refolding quality control system, including homologues of UGGT and α- and β-subunits of glucosidase II. The null mutant generated in this study confirms the biochemical activity of glucosidase II, since in its absence half of the N-linked glycans of VSG are expressed bearing terminal αGlc residues on their 3-arms. This also infers that the UGGT homologue is expressed in bloodstream form T. brucei and is enzymatically active.

Although bloodstream form T. brucei makes Man,GlcNAc2,PP-Dol (21, 22), Bangs et al have shown that one of the two N-glycosylation sites of VSG variant ILTat.1.3 receives an Endo H-resistant glycan in the ER (23). This observation, reproduced here for VSG221 (Fig. 1), together with the identification by Zamze et al (4) of unusually small (Man₉GlcNAc₂) N-glycans on Asn263 of mature VSG221 and the accumulation of substantial amounts of Man,GlcNAc₂,PP-Dol in bloodstream form T. brucei (24), suggests two models of VSG N-glycosylation in the ER of bloodstream form T. brucei: (a) The transfer of Man,GlcNAc₂ to some N-glycosylation sites and the transfer of biantenary (Endo H-resistant) Man,GlcNAc₂ to others. (b) The transfer of Man,GlcNAc₂ to both sites and the unusually rapid (immediate) processing of some sites to Endo H-resistant structures in the ER. We reasoned, based on the specificity of UGGT for Man₉,GlcNAc₂ linked to unfolded protein in other organisms (13), that deletion of the TbgEIIα glucosidaseII would ‘lock’ protein-linked Man,GlcNAc₂ structures in a glucosylated form and allow us to discriminate between these two models. Thus, we expected to find either, according to model (a), Glc₄Man₉,GlcNAc₂ at the Asn428 site of VSG221 and unchanged small Endo H-resistant structures at the Asn263 site or, according to model (b), Glc₄Man₉,GlcNAc₂ at both sites. However, analysis of the oligosaccharide structures attached to the two N-glycosylation sites of VSG221 in the null mutant revealed unexpected features. Thus, the C-terminal Asn428 N-glycosylation site was unchanged (carrying predominantly Man,GlcNAc₂ and Man,GlcNAc₃) whereas the internal Asn263 site now expressed larger structures due to the presence of an extended (αGlc-capped) 3-arm (Fig. 9). These results are counterintuitive since the preferred substrate for rat glucosidase II is Glc₄Man,GlcNAc₂, with
Glc₃Man₃GlcNAc₂ being a particularly poor substrate (40). Furthermore, the results suggest that *T. brucei* UGGT glucosylates Man₃GlcNAc₂, and not Man₃GlcNAc₂, on VSG whereas UGGT from other organisms has been reported to have a marked preference for Man₃GlcNAc₂ over Man₃GlcNAc₂ (13). These unexpected results prompted us to re-evaluate our assumptions, based on examples from mammalian cells (41), that Man₃GlcNAc₂ structures attached to protein in the ER of the parasite could not be processed by ER mannosidases to anything other than triantennary H-sensitive Man₃GlcNAc₂ structures. Consequently, we analysed the effects of a cocktail of three potent cell-permeable α-mannosidase inhibitors (kifunensin, swainsonine and 1-deoxymannojirimycin), which between them can inhibit all known classes of ER and Golgi α-mannosidases, on the processing of wild-type VSG221 N-glycans. In these experiments (Fig. 8), the inhibitors were clearly active but the results were consistent with model (a), above.

Thus, we postulate that *T. brucei* uses two types of dolichol-linked oligosaccharide for transfer to VSG in the ER (Fig. 10); a conventional triantennary Man₃GlcNAc₂-PP-Dol for transfer to sites destined to remain oligomannose (Man₃GlcNAc₂ to Man₃GlcNAc₂) and a biantennary Endo H-resistant Man₃GlcNAc₂-PP-Dol for transfer to sites destined to be directly involved in calreticulin-mediated protein folding and ultimately to be processed down to Man₃GlcNAc₂ and thence to complex structures. The fact that this organism uses these mechanisms on different sites of the same glycoprotein suggests that either the different VSG glycosylation sites somehow recruit different dolipol-PDol-oligosaccharide donors to the same ER translocon/OST complex or that one or both sites are glycosylated after protein translocation into the ER. In the latter model, the different VSG glycosylation sites might then recruit OST complexes with different donor specificities. The fact that post-translational N-glycosylation has been observed for some VSG variants (23, 42) provides tentative support for the latter model.

While it has been shown that other eukaryotes will transfer structures other than Glc₃Man₃GlcNAc₂ to protein (eg. many other trypanosomatids transfer Man₃GlcNAc₂, Man₃GlcNAc₂ or Man₃GlcNAc₂ (14) and the protozoan *Tetrahymena pyriformis* (43) and glucose-starved baby hamster kidney cells (44) transfer Glc₃Man₃GlcNAc₂) this is, to our knowledge, only the second example (after *T. cruzi* bloodstream trypomastigotes (14, 19)) of an organism utilising more than one Dol-PP-oligosaccharide donor under normal growth conditions and the first example of site-specific N-glycosylation in a single glycoprotein.

In *T. brucei*, the requirement for such a dual system of protein N-glycosylation may stem from the requirements for rapid of VSG synthesis (10 million copies must be made for each cell division). Thus, once the trypanosomatid lost the strict requirement for glucosylated Dol-PP-oligosaccharides for efficient transfer to protein by OST, they would have also lost the requirement to process down from Man₃GlcNAc₂ to Man₃GlcNAc₂ prior to adding additional sugars. Since, according to precedent (7, 10, 11), the smallest Dol-PP-oligosaccharide to appear in the lumen of the ER is likely to be biantennary Man₃GlcNAc₂-PP-Dol it makes metabolic sense that *T. brucei* should immediately attach this structure to those N-glycosylation sites destined for processing to Man₃GlcNAc₂, Man₃GlcNAc₂ and conventional Man₃GlcNAc₂-based complex structures. Indeed, such a mechanism still allows the transfer and processing of Man₃GlcNAc₂ to conventional (Man₃αGlcNAc₂) oligomannose structures where these are required but minimises the drain on Dol-P-Man donors (necessary for converting Man₃GlcNAc₂ to Man₃GlcNAc₂) that are also required for GPI anchor biosynthesis. The latter is prodigious in these organisms because they appear to make >10-fold excess of GPI precursors over their requirement for VSG anchorage (45).

Finally, it is worth noting that analysis of the VSG of the *TbGlcaset1a* null mutant has also revealed significant differences between the parasite and higher eukaryotes in N-glycan processing to complex structures. Whereas in higher eukaryotes the addition of β1-2 linked GlcNAc residues via GnT-I and GnT-II to the 3- and 6-arms, respectively, of the Man₃GlcNAc₂ core occurs by GnT-I action on the triantennary acceptor substrate Man₃GlcNAc₂, followed removal of two αMan residues and the action of GnT-II on the GlcNAcβ1-2Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc product (46), this sequence of events does not occur in *T. brucei*. For example, in the *TbGlcaset1a* null mutant it is evident that biantennary Glc₃Man₃GlcNAc₂ can act as a substrate for TbGnT-II (and subsequently for β1-4Gal-T) on the 6-arm. Also, since wild type trypanosomes normally process the glycan at Asn263 up to a conventional biantennary complex structure (Fig. 9), it appears that the substrate for TbGnT-I is Man₃GlcNAc₂ and not triantennary Man₃GlcNAc₂. We may, therefore, conclude that the GlcNAc-transferases involved in making complex N-glycans in *T. brucei* are different from their mammalian counterparts. Indeed, BLAST searches for homologues of the mammalian enzymes fail to return obvious parasite homologues. Such differences in amino acid sequence and substrate specificity point to host/parasite differences in N-glycan processing enzymes that may be exploitable.

In summary, the results presented here support the conclusions that: (i) Bloodstream form *T. brucei* can transfer both Man₃GlcNAc₂ and Man₃GlcNAc₂ to VSG in a site-specific manner. (ii) Unlike organisms that transfer exclusively Glc₃Man₃GlcNAc₂, the *T. brucei* UGGT and glucosidase II enzymes appear to prefer Man₃GlcNAc₂ Glc₃Man₃GlcNAc₂, respectively, as their substrates (though data from more glycoprotein glycosylation sites is needed to test this hypothesis). (iii) The ability to transfer Man₃GlcNAc₂ structures to N-glycosylation sites destined to become Man₃αGlcNAc₂ or complex structures may have evolved as a mechanism to conserve Dol-P-Man donors for GPI anchor biosynthesis in this organism. (iv) There are fundamental differences in the acceptor substrate specificities of host and parasite GlcNAc-transferases that initiate the synthesis of complex N-glycans.
REFERENCES

The abbreviations used are: CNX, calnexin; CRT, calreticulin; Dol, dolichol; Dol-P, dolichyl pyrophosphate; Endo H, endoglycosidase H; ER, endoplasmic reticulum; ES-MS, electrospray-mass spectrometry; ES-MS/MS, electrospray-mass spectrometry-mass-spectrometry; GPI, glycosylphosphatidylinositol; HPAC, high-pH anion exchange chromatography; HPT, hygromycin phosphotransferase; HPTLC, high-performance thin layer chromatography; OST, oligosaccharyltransferase; PAM, partially methylated alditol acetate; PNGase F, peptide N-glycosidase F; UGGT, UDP-Glc : glycoprotein glucosyltransferase; VSG, variant surface glycoprotein.

FOONOTES

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Fig. 1. Endo H-resistant N-glycans are added to one site of VSG221 in the endoplasmic reticulum.
Fluorograph of an SDS-PAGE gel of lysates of bloodstream form T. brucei cells. The cells were pulse-labelled for 3 min with [35S]-methionine. Lysates were mock treated (lanes 1 and 3) or treated with Endo H (lane 2) or PNGase F (lane 4). The apparent molecular weights and glycosylation status of the VSG221 bands are indicated on the left.

Fig. 2. Construction of a TbGlcαIα null mutant trypanosome.
Panel A: Summary of the of homologous recombination gene replacement events with hygromycin phosphotransferase (HPT) and puromycin acetyltransferase (PAC) drug-resistance genes. Panel B: Southern blot of genomic DNA digested with PstI from wild-type cells (lane 1), ΔTbGlcαIα::HPT (single-allele replacement) cells (lane 2) and ΔTbGlcαIα::HPT / ΔTbGlcαIα::PAC null mutant cells (lane 3). The blot was probed a 5′-UTR probe and indicates the replacement of both alleles with drug resistance genes.

Fig. 3. Mass spectrometric analysis of intact sVSG221 from wild-type, TbGlcαIα null and 1-deoxynojirimycin treated trypanosomes.
Samples of whole sVSG, from small-scale VSG preparations, were analysed by ES-MS on a QTof2 instrument and the spectra deconvolved by maximum entropy. The sVSG samples were from wild-type cells (panel A), the TbGlcαIα null mutant (panel B), the induced TbGlcαIα conditional null (‘add-back’) mutant (panel C) and wild-type cells grown in the presence of the glucosidase inhibitor 1-deoxynojirimycin (panel D).

Fig. 4. Mass spectrometric analysis of Pronase glycopeptides from sVSG221 from wild-type and TbGlcαIα null mutant trypanosomes.
Pronase digests of wild-type (panel A) and TbGlcαIα null mutant (panel B) sVSG221 were analysed by ES-MS/MS of individual ions. Representative daughter ion spectra are shown for the [M+2H]2 Ser-GPI C-terminal glycopeptide ion at m/z 1049.7 (panel D); the [M+2H]2 Hex,GlcNAc2,Asn-Thr glycopeptide ion at m/z 810.7 (panel E); the [M+2H]2 Hex,GlcNAc2,Asn-Glu-Thr glycopeptide ion at m/z 871.1 (panel F).

Fig. 5. 1H-NMR analysis of the Hex4HexNAc2 and Hex6HexNAc3 glycans isolated from the Asn286 site of sVSG221 from TbGlcαIα null mutant trypanosomes.
Panel A: Anomeric region of the 1-D 1H-NMR spectrum of the Hex4HexNAc2 glycan. The H-1 proton resonances were assigned to residue types (αGlc, αMan, βMan, βGlcNAc) according to J1,2 coupling constants, chemical shifts and the chemical shifts of adjacent protons in the sugar rings deduced from 2D-COSY spectra (Table III). Panel B: Reporter region of the ROESY spectrum of the Hex4HexNAc2 glycan showing intra- and inter-residue connectivities. The boxed crosspeaks indicate the through-space connectivities that suggest the sequence Glcα1-3Manα1-Mamα1-2Manα1-..
Residue descriptors are those shown in the box in panel A. Panel C: Anomeric region of the 1-D 1H-NMR spectrum of the Hex6HexNAc3 glycan. The H-1 proton resonances were assigned to residue types (αGlc, αMan, βMan, βGlcNAc)
Fig. 6. Mass spectrometric analysis of glycans isolated from the Asn263 site of sVSG221 from *Th. GlcaseIIα* null mutant trypanosomes after permethylation.

Permethylated glycans were analysed by daughter ion ES/MS. Panel A: Daughter ion spectrum of the [M+2Na]2+ permethylated Hex,HexNAc, ion. Panel B: Daughter ion spectrum of the [M+2Na]2+ permethylated Hex,HexNAc3 ion. Panel C: Daughter ion spectrum of the [M+2Na]2+ permethylated Hex,HexNAc3 ion. All spectra were processed (using the MassLynx maximum entropy 3 algorithm) to produce spectra consisting only of singly-charged ions.

Fig. 7. HPTLC analysis of NaB'H4-reduced glycans from the Asn263 site of sVSG221 from *Th. GlcaseIIα* null mutant trypanosomes.

The Hex,HexNAc, Hex,HexNAc, and Hex,HexNAc, glycans were labelled with tritium by NaB'H4-reduction and analysed by HPTLC and fluorography. Panel A: Labelled glycans were analysed before (-) and after (+) partial acetylation, as indicated, to preferentially cleave Manα1-6Man glycosidic bonds. The interpretation of the intermediate and final products (labelled a-d) are shown in (panel C). The control glycan (conventional Man,GlcNAc,) produced intermediate and final products of Man,GlcNAc (y) and Man,GlcNAc (x), as expected (lanes 7 and 8). Panel B: Labelled glycans were analysed before (-) and after (+) digestion with *E.coli* β1-4 galactosidase. The interpretation of the product of the digestion of Hex,HexNAc, is shown inside the dotted box in (panel C). The control biantennary glycan (NA2) lost two terminal βGal residues, as expected (lanes 9 and 10). The control Thy-1 GPI glycan was unaffected by β-galactosidase digestion (lanes 11 and 12), showing that the enzyme was not contaminated with α-mannosidase or β-N-acetylhexosaminidase activities.

Fig. 8. The effects of α-mannosidase inhibitors on VSG221 N-glycosylation.

Panel A: Fluorogram of an SDS-PAGE gel of lysates of bloodstream form *T. brucei* cells. The cells were pre-incubated with a cocktail of α-mannosidase inhibitors and pulse-labelled for 3 min with [35S]-methionine. Lysates were mock treated (lanes 1 and 3) or treated with Endo H (lane 2) or PNGase F (lane 4). The apparent molecular weights and glycosylation status of the VSG21 bands are indicated on the left. Panels B and C: Samples of whole sVSG from untreated (panel B) and α-mannosidase inhibitor-treated (panel C) trypanosomes were analysed by ES-MS on a Q-StarXL instrument and the spectra deconvolved by Bayesian protein reconstruction. Panels D and E: Pronase digests of sVSG from untreated (panel D) and α-mannosidase-treated (panel E) trypanosomes were analysed by ES-MS/MS in neutral loss scanning mode (neutral loss of m/z 81) to identify doubly-charged glycopeptide ions. The likely identities of the ions, based on measured mass, are indicated. These assignments were supported by daughter ion scanning ES-MS/MS of individual ions (data not shown). Ions marked with a black triangle belong to Ser-GPI fragments. The N-linked glycopeptides appear as multiple ions due to incomplete Pronase digestion. Thus, the Asn428 site is found as NE- and NET-containing glycopeptides.

Fig. 9. VSG221 glycosylation in wild-type and *Th. GlcaseIIα* null mutant trypanosomes.

The boxed structures show the main N-glycan species found at each N-glycosylation site of mature VSG221 from wild-type (4), *Th. GlcaseIIα* null mutant trypanosomes and α-mannosidase inhibitor treated trypanosomes (this study).

Fig. 10. Summary of the similarities and differences between general eukaryote protein N-glycosylation and processing systems (panel A) and those of bloodstream form *T. brucei* (panel B).
Table I. BLAST search results for *T. brucei* genes with homology to yeast and mouse genes associated with the dolichol cycle and early events of protein N-glycosylation in the ER.

tBLASn searches (33) with yeast (*S. cerevisiae*) and mouse (*M. musculus*) protein sequences were used to identify putative *T. brucei* homologues. The percentages of amino acid identity, amino acid similarity and the bit scores are indicated.

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a Searching with ALG5 returns the same gene as querying with DPM1. The reported subject shows greater homology to DPM1.
b BLAST search using yeast / mouse query identifies five predicted genes all annotated as ER mannosidase, only two are indicated here.
Table II. Isobaric glycoforms of sVSG221 detected by ES-MS.

The molecular weights of different glycoforms of sVSG221 were calculated according to the indicated compositions. The -, tr, +, ++, and +++ scores indicate the relative abundances of those glycoforms observed in (Fig. 3) for the sVSG221 preparations from the different cell lines, i.e., wild type cells (wild type), \(TbGlcase\) α null mutant cells (Null mutant), induced conditional null mutant cells (Add-back) and wild type cells treated with 1-deoxynojirimycin (dNJ).

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\(a\)Protein MW is based on the amino acid sequence of the VSG221 precursor (accession number P26332) minus residues 1-27 (signal peptide) and 460-476 (GPI attachment signal peptide) and allows for 4 disulphide bonds (MW = 46284 Da).

\(b\)Components specific to the GPI anchor and common to all glycoforms; I-C = 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 wild-type sVSG221 is expected to contain a GPI anchor of composition of Man\(_3\)Gal\(_5\) (35), a C-terminal N-linked glycan of Man\(_9\)GlcNAc\(_2\) and an internal N-linked glycan of Man\(_3\)GlcNAc\(_2\) (4) (i.e., GlcNAc = 4 and Man+Gal = 20).
Table III. Proton NMR Assignments for the Sugar Residues of the Hex$_6$HexNAc$_2$ and Hex$_6$HexNAc$_3$ Glycans from Asn263 of VSG221 from *TbGlc3a* Null Mutant Trypanosomes.

Chemical shift assignments for C-H protons of the Hex$_6$HexNAc$_2$ and Hex$_6$HexNAc$_3$ glycans were determined by COSY and TOCSY 2D-$^1$NMR. Correlations were used to assign protons starting from the well-resolved H1 anomic proton region. Figures in brackets belong to the indicated residue spin-system but cannot be assigned to specific positions.

Chemical Shift Assignments for Hex$_6$HexNAc$_2$

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Table IV. GC-MS Methylation Linkage Analysis of Hex$_6$HexNAc$_2$ and Hex$_6$HexNAc$_3$ Glycans from Asn263 of VSG221 from *TbGlaceII*α Null Mutant Trypanosomes.

The purified Hex$_6$HexNAc$_2$ and Hex$_6$HexNAc$_3$ glycans were permethylated, hydrolysed, deutero-reduced and acetylated to yield partially methylated alditol acetates (PMAAs) for analysis by GC-MS. Residue types were deduced from the electron-impact mass spectra and retention times.

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</tr>
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<td>[1-$^2$H]-1,5-di-O-acetyl-2-N-methylacetamido-3,4,6-tri-O-methyl-glucosaminitol</td>
<td>t-GlcNAc</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>[1-$^2$H]-1,4,5-tri-O-acetyl-2-methylacetamido-3,6-di-O-methyl-glucosaminitol</td>
<td>4-GlcNAc</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. 1

- 57 kDa (2 N-glycans)
- 54.5 kDa (1 N-glycan)
- 53 kDa (0 N-glycan)

Endo H
- 1
+ 2
- 3
- 4

PNGase F
- 1
- 2
- 3
+ 4
Fig. 2.
Fig. 3
Fig. 6.
Fig. 7
Fig. 8

A

57 kDa (2 N-glycans)
54.5 kDa (1 N-glycan)
53 kDa (0 N-glycans)

Endo H
PNGase F

B

Relative intensity vs. mass (Da)

C

Relative intensity vs. mass (Da)

D

E

Man9GlcNAc2
Man8GlcNAc2
Man7GlcNAc2
Man6GlcNAc2
Man5GlcNAc2
Man4GlcNAc2
Man3GlcNAc2
Man2GlcNAc2
ManGlcNAc2

Man9GlcNAc2
Man8GlcNAc2
Man7GlcNAc2
Man6GlcNAc2
Man5GlcNAc2
Man4GlcNAc2
Man3GlcNAc2
Man2GlcNAc2
ManGlcNAc2

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Fig. 9.

Wild-type VSG221

TbGlcαaseIIα null VSG221

Plus mannosidase inhibitors VSG221
Fig. 10.
Deuan C. Jones, Angela Mehlert, M. Lucia S. Güther and Michael A. J. Ferguson

J. Biol. Chem. published online August 24, 2005

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