

# The lipid-linked oligosaccharide donor specificities of *Trypanosoma brucei* oligosaccharyltransferases

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We recently presented a model for site-specific protein N-glycosylation in *Trypanosoma brucei* whereby the TbSTT3A oligosaccharyltransferase (OST) first selectively transfers biantennary Man<sub>5</sub>GlcNAc<sub>2</sub> from the lipid-linked oligosaccharide (LLO) donor Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol to N-glycosylation sequons in acidic to neutral peptide sequences and TbSTT3B selectively transfers triantennary Man<sub>9</sub>GlcNAc<sub>2</sub> to any remaining sequons. In this paper, we investigate the specificities of the two OSTs for their preferred LLO donors by glycotyping the variant surface glycoprotein (VSG) synthesized by bloodstream-form *T. brucei* *TbALG12* null mutants. The *TbALG12* gene encodes the  $\alpha$ 1-6-mannosyltransferase that converts Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol to Man<sub>8</sub>GlcNAc<sub>2</sub>-PP-Dol. The VSG synthesized by the *TbALG12* null mutant in the presence and the absence of  $\alpha$ -mannosidase inhibitors was characterized by electrospray mass spectrometry both intact and as pronase glycopeptides. The results show that TbSTT3A is able to transfer Man<sub>7</sub>GlcNAc<sub>2</sub> as well as Man<sub>5</sub>GlcNAc<sub>2</sub> to its preferred acidic glycosylation site at Asn263 and that, in the absence of Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol, TbSTT3B transfers both Man<sub>7</sub>GlcNAc<sub>2</sub> and Man<sub>5</sub>GlcNAc<sub>2</sub> to the remaining site at Asn428, albeit with low efficiency. These data suggest that the preferences of TbSTT3A and TbSTT3B for their LLO donors are based on the c-branch of the Man<sub>9</sub>GlcNAc<sub>2</sub> oligosaccharide, such that the presence of the c-branch prevents recognition and/or transfer by TbSTT3A, whereas the presence of the c-branch enhances recognition and/or transfer by TbSTT3B.

**Keywords:** N-glycosylation / oligosaccharyltransferase / STT3 / *Trypanosoma brucei*

## Introduction

The African trypanosomes are tsetse fly-transmitted protozoan parasites. Two lifecycle stages are most amenable to laboratory cultivation: the procyclic form that normally grows in the tsetse fly midgut and the bloodstream form that causes African sleeping sickness in humans and nagana in cattle. The procyclic form of the parasite presents a coat that includes a set of glycosylphosphatidylinositol (GPI)-anchored glycoproteins known as procyclins, characterized by internal dipeptide (EP) or pentapeptide (GPEET) repeats (Roditi et al. 1989; Treumann et al. 1997), free GPI structures (Lillico et al. 2003; Vassella et al. 2003; Nagamune et al. 2004; Roper et al. 2005) and a high-molecular-weight glycoprotein complex (Guther et al. 2009). Most EP procyclins contain a single N-glycosylation site, occupied exclusively by a conventional triantennary Man<sub>5</sub>GlcNAc<sub>2</sub> oligomannose oligosaccharide at the N-terminal side of the EP-repeat domain (Acosta-Serrano et al. 1999). The bloodstream form of the parasite is covered in a densely packed layer of  $5 \times 10^6$  GPI-anchored variant surface glycoprotein (VSG) dimers. This coat protects the parasites from the alternative pathway of complement-mediated lysis, shields other cell surface proteins from the host immune system and, by the process of antigenic variation, allows the parasites to persist indefinitely in the host bloodstream (Cross 1996; Pays and Nolan 1998). The trypanosome genome contains several hundred silent VSG genes, some of which are pseudogenes, and antigenic variation involves the switching of the expression of these genes, which encode immunologically distinct GPI-anchored glycoproteins with 1–3 N-glycosylation sites (Mehlert et al. 1998; Hutchinson et al. 2003; Marcello and Barry 2007). The bloodstream-form parasite also expresses other less abundant GPI-anchored and/or N-glycosylated glycoproteins that are arranged either randomly in the VSG coat, like the invariant glycoproteins ISG65 and ISG75 (Ziegelbauer and Overath 1992), or in specific surface locations such as Fla1 which is localized to the flagellar adhesion zone (Nozaki et al. 1996) and the transferrin receptor which is localized to the flagellar pocket (Steверding et al. 1994). Other glycoproteins are located primarily in intracellular sites, like lysosomal p67 (Kelley et al. 1999), Golgi and lysosomal tGLP1 (Lingnau et al. 1999), endoplasmic reticulum (ER) GPIdeAc (Guther et al. 2003) and endosomal TbMBAP1 (Engstler et al. 2005). Although the procyclic form shares some of these glycoproteins, like p67, tGLP1 and Fla1, others are bloodstream-form specific, such as ISG65, ISG75, TbMBAP1 and the transferrin receptor.

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Lipid-linked oligosaccharide (LLO) donors for protein N-glycosylation are assembled in the membrane of the ER. Each of the sugars is added to the growing LLO by asparagine-linked glycosylation (ALG) glycosyltransferases, which are numbered according to the order of their discovery rather than by the sequence of enzymatic steps (Burda and Aebi 1999). Genomic and experimental comparisons have shown that some lower eukaryotes do not possess all the ALG genes needed to make the canonical mature Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol LLO structure typical of most eukaryotes (Parodi 1993; Samuelson et al. 2005). *Trypanosoma brucei*, for example, lacks the ALG8 and ALG10 glucosyltransferase genes (Jones et al. 2005; Samuelson et al. 2005) such that Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol is the largest LLO it can synthesize. Interestingly, the Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol intermediate is by far the most abundant LLO in procyclic and bloodstream-form parasites (Low et al. 1991; Acosta-Serrano et al. 2004; Manthri et al. 2008). Uniquely, *T. brucei* uses both Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol and Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol to N-glycosylate its glycoproteins (Jones et al. 2005; Manthri et al. 2008; Izquierdo, Schulz, et al. 2009) and this explains why endoglycosidase-H-resistant N-glycans can appear on *T. brucei* glycoproteins co-translationally, as first noted and discussed by Bangs et al. (1988).

The enzymes that transfer oligosaccharides from the LLOs are called oligosaccharyltransferases (OSTs). These are typically multi-subunit integral membrane protein complexes that mediate the *en bloc* transfer of the preassembled oligosaccharide onto asparagine in glycosylation sequons (mostly Asn-Xaa-Thr/Ser; Xaa ≠ Pro) of nascent polypeptides entering the lumen of the ER (Yan and Lennarz 2002, 2005; Kelleher et al. 2003; Kelleher and Gilmore 2006). However, prokaryotic (Lizak et al. 2011) and kinetoplastid OSTs consist of a single-subunit homologous to the STT3 catalytic subunits of OST complexes (Kelleher and Gilmore 2006). In the case *T. brucei*, OST activity is catalyzed by two single-subunit enzymes that selectively transfer distinct oligosaccharide donors to specific glycosylation sites (Jones et al. 2005; Manthri et al. 2008; Izquierdo, Schulz, et al. 2009). Thus, TbSTT3A first transfers biantennary Man<sub>5</sub>GlcNAc<sub>2</sub> from Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol to glycosylation sites in acidic to neutral regions of polypeptides and TbSTT3B transfers triantennary Man<sub>9</sub>GlcNAc<sub>2</sub> from Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol to remaining glycosylation sites (Izquierdo, Schulz, et al. 2009). The selective recruitment of triantennary Man<sub>9</sub>GlcNAc<sub>2</sub> or biantennary Man<sub>5</sub>GlcNAc<sub>2</sub> to specific glycosylation sites in this way is highly significant because it predetermines the kind of processing, and therefore the repertoire of final glycan structures, that can be generated at that site. Specifically, triantennary Man<sub>9</sub>GlcNAc<sub>2</sub> can only be processed as far as triantennary Man<sub>5</sub>GlcNAc<sub>2</sub> by *T. brucei*, which lacks a Golgi  $\alpha$ -mannosidase II gene, thus strictly limiting the glycoforms at such sites to the oligomannose series. In contrast, biantennary Man<sub>5</sub>GlcNAc<sub>2</sub> is the only route to paucimannose and complex N-glycans in the parasite (Manthri et al. 2008), including the novel giant poly-N-acetylglucosamine structures found in bloodstream-form *T. brucei* (Atrih et al. 2005). This model of sequon pI controlling the oligomannose vs. paucimannose/complex N-glycosylation fate at particular

N-glycosylation correctly predicted the N-glycan type attached to a previously uncharacterized *T. brucei* VSG (Mehlert et al. 2010).

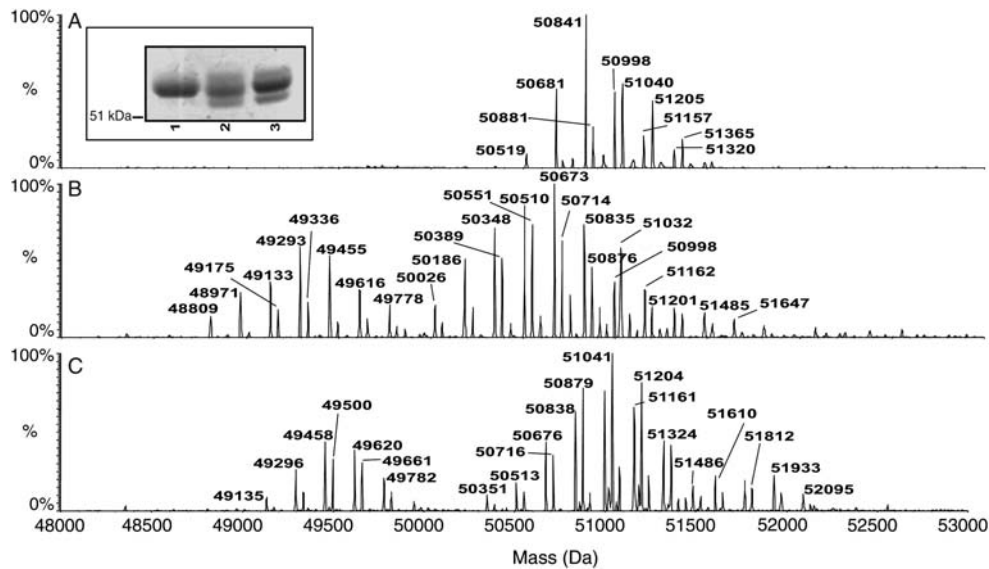
The *T. brucei* ALG12 gene (*TbALG12*) encodes a Dol-P-Man:Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol  $\alpha$ 1-6-mannosyltransferase. In the cultured form of the normally insect-dwelling (procyclic) life-cycle stage of the parasite, deletion of this gene reduced the maximum LLO size from Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol to Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol, as expected (Acosta-Serrano et al. 2004). The ALG12<sup>-/-</sup> cells proved to be resistant to the cytotoxic action of concanavalin A because they express procyclins with altered N-glycans, predominantly shorter paucimannose glycans (Man<sub>4</sub>GlcNAc<sub>2</sub>) with or without a terminal N-acetylglucosamine unit (Leal et al. 2004).

This paper describes the glycotyping of the VSG from bloodstream-form ALG12<sup>-/-</sup> mutants. The variant 221 (also known as MITat1.2) VSG coat glycoprotein is an excellent reporter because it contains two N-glycosylation sites: one at Asn263 occupied by small, biantennary paucimannose and complex structures, which originate from the transfer of Man<sub>5</sub>GlcNAc<sub>2</sub> by TbSTT3A from the Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol precursor, and one at Asn428 occupied by conventional triantennary oligomannose structures, which originate from the transfer of Man<sub>9</sub>GlcNAc<sub>2</sub> by TbSTT3B from Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol (Jones et al. 2005; Manthri et al. 2008; Izquierdo, Schulz, et al. 2009). Thus, analysis of VSG221 N-glycosylation allows us to simultaneously assess the effects of genetic or chemical perturbations on both mechanisms of protein N-glycosylation in this organism (Jones et al. 2005; Urbaniak et al. 2006; Manthri et al. 2008; Stokes et al. 2008; Izquierdo, Atrih, et al. 2009; Izquierdo, Schulz, et al. 2009).

## Results

### *Analysis of intact soluble-form VSG221 synthesized by TbALG12 null mutant cells*

Wild-type and *TbALG12*<sup>-/-</sup> mutant cell lines were grown in vitro, the latter in the presence and the absence of a cocktail of  $\alpha$ -mannosidase inhibitors (MI), and samples of  $2 \times 10^8$  cells from 100 mL of culture were processed to yield  $\sim 100$   $\mu$ g of purified soluble-form VSG221 (sVSG221). sVSG arises from the cleavage of diacylglycerol from the GPI anchor by the action of an endogenous GPI-specific phospholipase C (Cardoso de Almeida and Turner 1983; Cross 1984; Ferguson et al. 1985). The sVSG221 synthesized by the ALG12<sup>-/-</sup> parasites showed different glycosylation patterns to that synthesized by wild-type cells, as judged by SDS-PAGE (Figure 1, inset). Aliquots of the purified sVSGs were also analyzed by positive-ion electrospray-mass spectrometry (ES-MS). The deconvolved mass spectrum of intact wild-type sVSG221 (Figure 1A; Table I) showed the range of expected glycoforms that arise from the known heterogeneity in the GPI anchor and N-glycan sites (Zamze et al. 1991; Mehlert et al. 1998; Jones et al. 2005; Manthri et al. 2008; Izquierdo, Schulz, et al. 2009). In the case of sVSG221 synthesized by the ALG12<sup>-/-</sup> mutant in the absence of MI, the analysis revealed a group of glycoforms centered around 49,293 Da



**Fig. 1.** SDS-PAGE and mass spectrometric analyses of intact sVSG221 from wild-type and *ALG12*<sup>-/-</sup> null trypanosomes. The “inset” shows a Coomassie stained SDS-PAGE gel showing the migration of sVSG221 from wild-type cells (lane 1), the *ALG12*<sup>-/-</sup> null mutant (lane 2) and the *ALG12*<sup>-/-</sup> null mutant grown in the presence of MI (lane 3). The panels show the mass spectrometric analysis of sVSG221 from wild-type cells (A), the *ALG12*<sup>-/-</sup> null mutant (B) and the *ALG12*<sup>-/-</sup> null mutant grown in presence of MI (C).

similar to the underglycosylated sVSG221 glycoforms found in the *TbALG3*<sup>-/-</sup> mutant and in the UDP-GlcNAc pyrophosphorylase conditional null mutant under non-permissive conditions, i.e. with masses consistent with the C-terminal Asn428 N-glycosylation site being unoccupied (Urbaniak et al. 2006; Manthri et al. 2008; Stokes et al. 2008). The higher molecular weight group of glycoforms, centered around 50,673 Da, with both N-glycosylation sites occupied, showed a wider range of glycoforms than the wild-type profile, including glycoforms up to three hexose units smaller and some with significantly higher *N*-acetylhexosamine to hexose ratios than the wild-type glycoforms (Table I). This suggests that the latter group of glycoforms may be enriched in *N*-glycans with LacNAc structures at one or both sites. The effect of MI on the *ALG12*<sup>-/-</sup> null mutant sVSG221 showed a general increase in size for both groups of glycoforms, and the significance of this is described in the end of the results section.

#### Analysis of pronase glycopeptides from sVSG221 synthesized by *TbALG12* null mutant cells

To further probe the nature of the changes in sVSG221 glycosylation induced by the deletion of the *ALG12* gene, aliquots of wild-type and *TbALG12* null mutant sVSG221 samples (50 µg) were digested with pronase and the resulting glycopeptides were enriched and analyzed by ES-MS and ES-MS/MS in a positive-ion mode (Manthri et al. 2008). As expected, the data showed no changes in the masses of the VSG GPI-peptide fragments in *ALG12*<sup>-/-</sup> cells or in these same cells treated with MI (Figure 2; Supplementary data, Table SI). However, the presence of glycopeptides like Hex<sub>4</sub>HexNAc<sub>4</sub>-NTT and Hex<sub>7</sub>HexNAc<sub>6</sub>-NTT are noteworthy (at 1957.76 and 2688.12 Da, respectively), since they indicate that, in the absence of its preferred Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol

precursor, TbSTT3B is able to transfer biantennary Man<sub>5</sub>GlcNAc<sub>2</sub> and/or Man<sub>7</sub>GlcNAc<sub>2</sub> from their respective LLO precursors to the C-terminal Asn428 site, albeit with low efficiency as indicated by the underglycosylation apparent in Figure 1B and C. Evidence that both Man<sub>5</sub>GlcNAc<sub>2</sub> and Man<sub>7</sub>GlcNAc<sub>2</sub> can be transferred to Asn428 comes from the analysis of the pronase glycopeptides made from sVSG221 synthesized by in *ALG12*<sup>-/-</sup> cells in the presence of MI, where both Hex<sub>7</sub>HexNAc<sub>2</sub>-GNTNT at 2068.74 Da and Hex<sub>5</sub>HexNAc<sub>2</sub>-GNTNT at 1744.62 Da are apparent (Figure 2C).

The presence of glycopeptides like Hex<sub>7</sub>HexNAc<sub>2</sub>-RNET (2058.88 Da) and Hex<sub>8</sub>HexNAc<sub>2</sub>-RNET (2220.92 Da) in the *ALG12*<sup>-/-</sup> sVSG221 sample suggests that TbSTT3A can utilize biantennary Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol as well as Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol (Figure 2B). We presume that the Hex<sub>8</sub>HexNAc<sub>2</sub>-containing glycopeptides are due to glucosylation of the a-branch of Man<sub>7</sub>GlcNAc<sub>2</sub> by UGGT (Izquierdo, Atrih, et al. 2009), an assumption that is supported by their significantly greater intensity in the *ALG12*<sup>-/-</sup> sVSG221 from MI-treated cells, where the a-branch is protected from digestion by ER mannosidases.

## Discussion

Taken together, the data presented here and summarized in Figure 3 indicate that TbSTT3A can use Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol with about equal efficiency to Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol (the main substrate used in wild-type cells) and that TbSTT3B can also use Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol with about equal efficiency to Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol. However, in the case of TbSTT3B, the underglycosylation of sVSG221 in *TbALG3*<sup>-/-</sup> (Manthri et al. 2008) and



**Table I.** Isobaric glycoforms of sVSG221 detected by ES-MS

Protein <sup>a</sup>	I-cP <sup>b</sup>	GlcN <sup>b</sup>	EtNP <sup>b</sup>	HexNAc	Hex	Molecular mass (Da)	WT/ALG12 <sup>-/-</sup> /ALG12 <sup>-/-</sup> MI (Theo.)	WT	ALG12 <sup>-/-</sup>	ALG12 <sup>-/-</sup> MI
1	1	1	1	2	10	NA/48809/NA(48816)		-	+	-
1	1	1	1	2	11	NA/48971/NA(48978)		-	+	-
1	1	1	1	2	12	NA/49133/49135(49140)		-	+	Traces
1	1	1	1	3	11	NA/49175/49176(49181)		-	+	Traces
1	1	1	1	2	13	NA/49293/49296(49302)		-	++	+
1	1	1	1	3	12	NA/49336/49337(49343)		-	+	+
1	1	1	1	2	14	NA/49455/49458(49464)		-	++	++
1	1	1	1	3	13	NA/49497/49500(49505)		-	+	+
1	1	1	1	2	15	NA/49616/49620(49626)		-	+	+
1	1	1	1	3	14	NA/49659/49661(49667)		-	+	+
1	1	1	1	2	16	NA/49778/49782(49788)		-	+	+
1	1	1	1	3	15	NA/NA/49823(49829)		-	-	+
1	1	1	1	4	15	NA/50026/NA(50032)		-	+	-
1	1	1	1	4	16	NA/50186/NA(50194)		-	++	-
1	1	1	1	5	15	NA/50229/NA(50235)		-	+	-
1	1	1	1	4	17	NA/50348/50351(50356)		-	+++	+
1	1	1	1	5	16	NA/50389/50392(50397)		-	++	Traces
1	1	1	1	4	18	50519/50510/50513(50518)		Traces	+++	+
1	1	1	1	5	17	NA/50551/50555(50559)		-	+++	+
1	1	1	1	6	16	NA/50597/NA(50600)		-	+	-
1	1	1	1	4	19	50681/50673/50676(50680)		++	+++	++
1	1	1	1	5	18	50717/50714/50716(50721)		Traces	++	+
1	1	1	1	6	17	50769/50759/50756(50762)		Traces	+	Traces
1	1	1	1	4	20	50841/50835/50838(50842 <sup>c</sup> )		+++	+++	++
1	1	1	1	5	19	50881/50876/50879(50883)		+	++	+++
1	1	1	1	6	18	NA/50919/50918(50924)		-	+	+
1	1	1	1	4	21	50998/NA/51000(51004)		++	+	+++
1	1	1	1	5	20	51040/51032/51041(51045)		++	++	+++
1	1	1	1	6	19	NA/51081/51081(51086)		-	+	+
1	1	1	1	4	22	51157/NA/51161(51166)		+	+	++
1	1	1	1	5	21	51205/51201/51204(51207)		++	+	+++
1	1	1	1	6	20	51247/51244/51243(51248)		Traces	Traces	+
1	1	1	1	4	23	51320/NA/51324(51328)		+	+	++
1	1	1	1	5	22	51365/51364/51365(51369)		+	+	++
1	1	1	1	8	19	51487/51485/51486(51492)		Traces	+	+
1	1	1	1	7	21	NA/NA/51610(51613)		-	-	+
1	1	1	1	8	20	NA/51647/51651(51654)		-	+	+
1	1	1	1	7	22	NA/NA/51772(51775)		-	-	+
1	1	1	1	8	21	NA/51809/51812(51816)		-	Traces	+
1	1	1	1	7	23	NA/NA/51933(51937)		-	-	+
1	1	1	1	8	22	NA/NA/51973(51978)		-	-	+
1	1	1	1	7	24	NA/NA/52095(52099)		-	Traces	+

The molecular weights of different glycoforms of sVSG221 were calculated according to the indicated compositions. The -, traces, +, ++ and +++ scores indicate the relative abundances of those glycoforms observed in sVSG preparations from the different cell lines, i.e. wild-type cells (WT), ALG12<sup>-/-</sup> null mutant cells (ALG12<sup>-/-</sup>) and ALG12<sup>-/-</sup> null mutant cells growth with mannosidase inhibitors (ALG12<sup>-/-</sup> MI).

<sup>a</sup>Protein Mr is based on the amino acid sequence of the VSG221 precursor (accession no. P26332) minus residues 1–27 (signal peptide) and 460–476 (GPI attachment signal peptide) and allows for four disulfide bonds (Mr = 46,284).

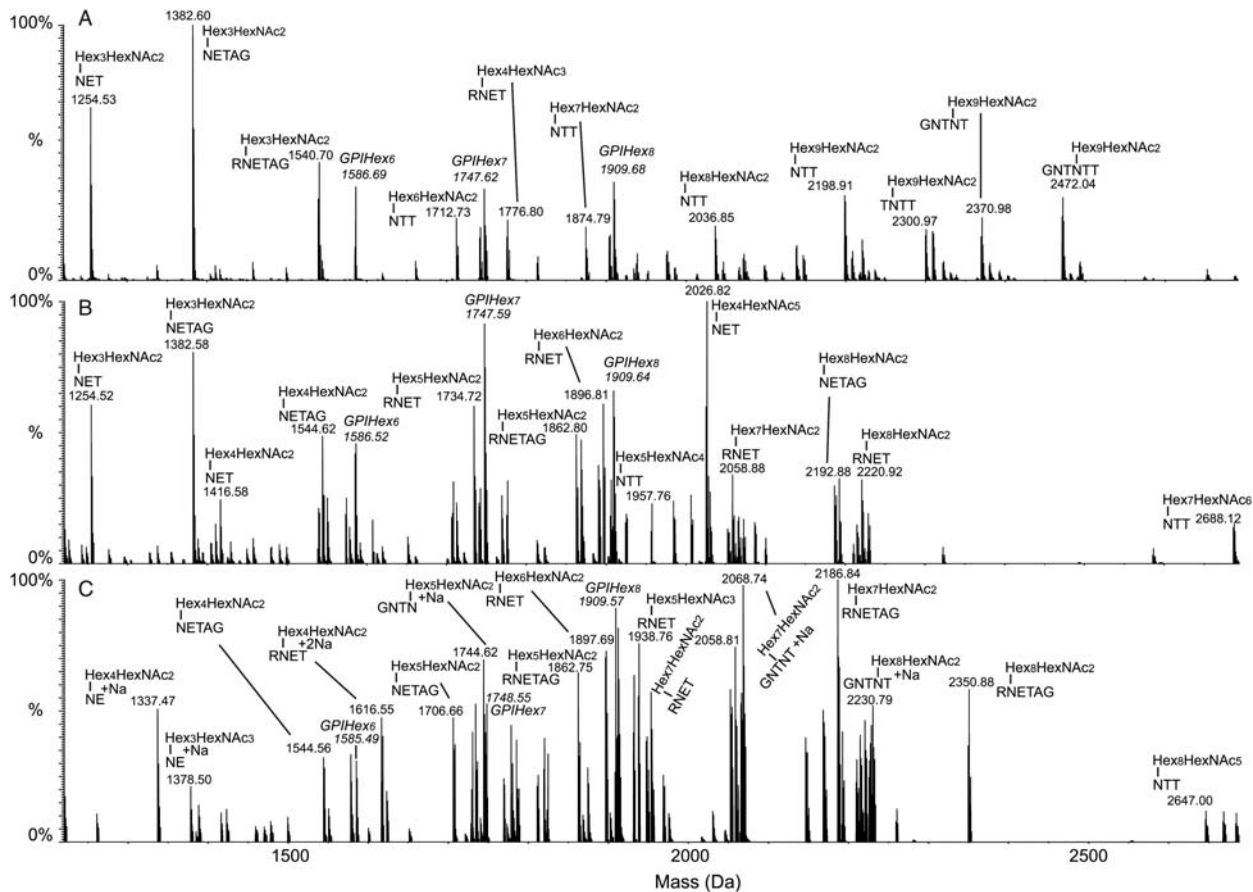
<sup>b</sup>Components specific to the GPI anchor and common to all glycoforms; I-cP *myo*-inositol-1,2 cyclic phosphate; EtNP, ethanolamine phosphate.

<sup>c</sup>The most abundant glycoform of wild-type sVSG221 is expected to contain a GPI anchor of composition of Man<sub>3</sub>Gal<sub>5</sub>, a C-terminal *N*-linked glycan of Man<sub>9</sub>GlcNAc<sub>2</sub> and an internal *N*-linked glycan of Man<sub>3</sub>GlcNAc<sub>2</sub>; i.e. HexNAc (GlcNAc) = 4 and Hex (Man + Gal) = 20.

*Tb*ALG12<sup>-/-</sup> cells (this paper) suggests that neither are the preferred LLO donor for TbSTT3B, which appears to have a strong preference for Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol. This, in turn, suggests that TbSTT3B requires the c-branch of the LLO oligosaccharide for efficient LLO recognition and/or transfer, whereas TbSTT3A does not; indeed, its presence may impede LLO recognition and/or transfer by TbSTT3A. This model of LLO selection by TbSTT3s would be in agreement to the mechanisms suggested for OSTs in other protists, such as *Trypanosoma cruzi*, *Entamoeba histolytica* and *Trichomonas vaginalis* (Kelleher et al. 2007). In these examples, a terminal  $\alpha$ 1,2-linked mannose residue on the b- or c-branch of Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol is a positive determinant for substrate

selection by the *T. cruzi* OST, whereas *E. histolytica* and *T. vaginalis* OSTs select donors with a non-glucosylated a-branch in the LLO but do not discriminate between Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol and Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol (Kelleher et al. 2007).

In summary, the underlying mechanism for site-specific N-glycosylation in *T. brucei* appears to be defined by the selectivity of TbSTT3B for LLO donors containing the c-branch and of TbSTT3A for LLO donors lacking the c-branch, coupled with the co-translational action of TbSTT3A and its selectivity for sequons in relatively acidic environments and the post-translational action of TbSTT3B with broad specificity for sequon environment (Izquierdo, Schulz, et al. 2009).

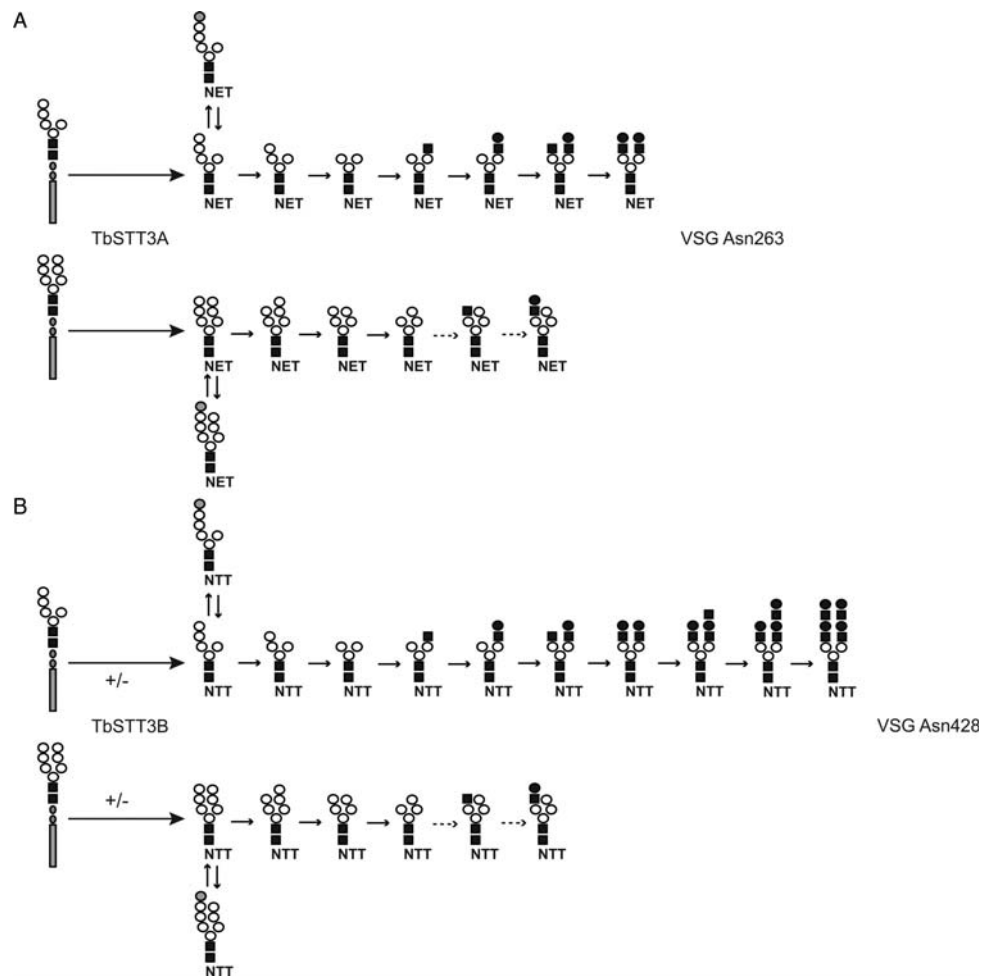


**Fig. 2.** Mass spectrometric analyses of pronase N-glycopeptides and GPI peptides. Aliquots of sVSG221 were digested with pronase and the glycopeptides were purified and analyzed by ES-MS in the positive-ion mode. The ES-MS spectra were processed using the Bayesian peptide reconstruction program in the ABI Analyst Software. The identities of the glycopeptides from wild-type (**A**), the *ALG12*<sup>-/-</sup> null mutant (**B**) and the *ALG12*<sup>-/-</sup> null mutant grown in presence of MI (**C**) are indicated (see also Supplementary data, Table SI) and were confirmed by MS/MS of the corresponding doubly charged ions (data not shown). GPI glycopeptides (which do not change between the different cell lines) are labeled in italics.

Alternatively, it could be argued that TbSTT3B absolutely requires the complete Man<sub>9</sub>GlcNAc<sub>2</sub> donor for activity and that the partial glycosylation observed in Asn428 is catalyzed entirely by TbSTT3A using either Man<sub>7</sub>GlcNAc<sub>2</sub>-PP-Dol or Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol, albeit inefficiently due to the absence of an optimal acidic-neutral polypeptide region. However, this latter model seems unlikely since it is known that, in the absence of TbSTT3B, TbSTT3A barely modifies Asn428 at all (Izquierdo, Schulz, et al. 2009) and, in contrast, there is a significant amount (>70%) of VSG with both N-glycosylation sites occupied in the *ALG12*<sup>-/-</sup> mutant cells (Figure 1B). Our results, therefore, support the notion that protists that cannot synthesize the canonical Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol LLO, such as *T. cruzi* and *E. histolytica*, have less stringent LLO donor specificity than organisms that do synthesize Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol, such as *Saccharomyces cerevisiae* and mammals (Kelleher et al. 2007).

The processing of the biantennary Man<sub>7</sub>GlcNAc<sub>2</sub> structure in bloodstream-form trypanosomes described in this paper, together with those previously reported for procyclic trypanosomes (Hwa et al. 1999; Hwa and Khoo 2000; Leal et al.

2004) and for the processing of biantennary Man<sub>5</sub>GlcNAc<sub>2</sub> in bloodstream-form and procyclic-form trypanosomes (Manthri et al. 2008), provides some insights into the specificities of the *T. brucei* UDP-GlcNAc:glycoprotein GlcNAc transferase type I (TbGnTI) and type II (TbGnTII) activities. These activities add βGlcNAc residues in 1-2-linkage to the 3- and 6-arm, respectively, of the Man<sub>α</sub>1-3(Man<sub>α</sub>1-6)Man<sub>β</sub>1-4GlcNAc<sub>β</sub>1-4GlcNAc (Man<sub>3</sub>GlcNAc<sub>2</sub>) core and can therefore be considered equivalent to the GnTI and GnTII activities found in other eukaryotes. However, the *T. brucei* genome does not contain any obvious candidate genes for these activities, although there are some 21 genes encoding putative UDP-GlcNAc/UDP-Gal-dependent glycosyltransferases of unknown function in the genome (Izquierdo, Nakanishi, et al. 2009). Given the apparent sequence disparity between general eukaryotic GnTI and GnTII enzymes and the parasite equivalents, one might also expect specificity differences, and this does appear to be the case: first, whereas GnTI from higher eukaryotes acts on Man<sub>α</sub>1-3(R-Man<sub>α</sub>1-6)Man<sub>β</sub>1-4GlcNAc<sub>β</sub>1-4GlcNAc, where R = H, Man<sub>α</sub>1-3 or Man<sub>α</sub>1-3(Man<sub>α</sub>1-6) (Kornfeld and Kornfeld 1985), TbGnTI



**Fig. 3.** A model of *N*-glycan processing in *ALG12*<sup>-/-</sup> null *T. brucei* cells. The solid long arrows illustrate the transfer of Man<sub>5</sub>GlcNAc<sub>2</sub> and Man<sub>7</sub>GlcNAc<sub>2</sub> from their respective LLOs to each of the two *N*-glycosylation sites of VSG221 in *ALG12*<sup>-/-</sup> null mutant trypanosomes. The +/- symbol indicates inefficient oligosaccharide transfer of Man<sub>7</sub>GlcNAc<sub>2</sub> and Man<sub>5</sub>GlcNAc<sub>2</sub> by TbSTT3B that normally transfers Man<sub>9</sub>GlcNAc<sub>2</sub>. The short solid arrows indicate the processing of Man<sub>5</sub>GlcNAc<sub>2</sub> and Man<sub>7</sub>GlcNAc<sub>2</sub> once attached to each of the VSG221 *N*-glycosylation sites. The dashed short arrows indicate the processing of atypical Man<sub>4</sub>GlcNAc<sub>2</sub> glycans to Man<sub>5</sub>GlcNAc<sub>3</sub> glycans as described in the analysis of concanavalin A-resistant procyclic mutants (Hwa and Khoo 2000).

does not appear to be able to work on substrates containing the underlined  $\alpha$ 1-6-linked Man residue. This means that *T. brucei* is incapable of making conventional hybrid *N*-glycans. Second, whereas the prior action of higher eukaryote GnTI is required for the subsequent action of GnTII, this is clearly not the case for TbGnTII. Indeed, the addition of  $\beta$ GlcNAc to the 6-arm of the Man $\alpha$ 1-3(Man $\alpha$ 1-6)Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc core by TbGnTII appears to be completely unaffected by the status of the 3-arm of the core, which can be unsubstituted or even substituted with mannobiose (i.e. Man $\alpha$ 1-2Man $\alpha$ 1-2Man $\alpha$ 1-3(Man $\alpha$ 1-6)Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc).

The aforementioned peculiarities in protein *N*-glycosylation and *N*-glycan processing in *T. brucei* have prompted us to consider protein *N*-glycosylation as a potential therapeutic target against African trypanosomes. This is further supported by the fact that both the TbSTT3A and TbSTT3B catalyzed branches of *N*-glycosylation to paucimannose/complex and oligomannose glycans, respectively, are essential for parasite growth in animals (Izquierdo, Schulz, et al. 2009).

## Materials and methods

### Cultivation of trypanosomes

Bloodstream-form *T. brucei* genetically modified to express T7 polymerase and the tetracycline repressor protein were cultivated in HMI-9 medium containing 2.5  $\mu$ g/mL of G418 at 37°C in a 5% CO<sub>2</sub> incubator as described in Wirtz et al. (1999). The *ALG12* null mutant strain was a generous gift from G. A. M. Cross lab (Leal et al. 2004).

### Small-scale sVSG isolation

sVSG was isolated from 100 mL cultures containing  $\sim 2 \times 10^8$  bloodstream-form *T. brucei*. The cultures were chilled in ice-water and centrifuged at 2500  $\times g$  for 10 min. The pellet was washed twice in trypanosome dilution buffer (Cross 1975) and transferred to a 1.5-mL Eppendorf tube. The pellet was resuspended in 300  $\mu$ L of lysis buffer (10 mM NaH<sub>2</sub>PO<sub>4</sub> buffer, pH 8.0, containing 0.1 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, 1  $\mu$ g/mL leupeptin and 1  $\mu$ g/mL aprotinin) prewarmed to 37°C and incubated for 5 min at the same

temperature. The sample was centrifuged at  $14,000 \times g$  for 5 min, and the supernatant was applied to a 200- $\mu$ L DE52 anion exchange column pre-equilibrated in lysis buffer. Fresh lysis buffer (800  $\mu$ L without protease inhibitors) was applied in four stages, and the pooled column eluate was concentrated and diafiltered with water on an YM-10 spin concentrator (Microcon, millipore, Watford, UK). The final sample of 50–100  $\mu$ g of sVSG221 was recovered in a volume of 100  $\mu$ L water.

#### ES-MS analysis of intact VSG

Samples of the sVSG preparations were diluted to  $\sim 0.07$   $\mu$ g/ $\mu$ L in 50% acetonitrile, 1% formic acid, loaded into nanotips (Micromass-type F) and analyzed by positive-ion ES-MS on a Q-Star XL instrument (Applied Biosystems, Paisley, UK). Data were collected and processed using the Bayesian protein reconstruction algorithm of Analyst software.

#### ES-MS and ES-MS/MS analysis of pronase glycopeptides

Aliquots of sVSG ( $\sim 50$   $\mu$ g in 50  $\mu$ L water) were mixed with 5  $\mu$ L of 1 M ammonium bicarbonate and 10  $\mu$ L of 1 mg/mL pronase in 5 mM calcium acetate and incubated at 37°C for 36 h. The pronase glycopeptides were purified on Envicarb graphitized carbon microcolumns, which were prepared as follows: the contents of an Envicarb cartridge (Supelco, Poole, UK) were suspended in methanol and a bed of  $\sim 20$   $\mu$ L of graphitized carbon was packed into a 100- $\mu$ L C4 OMIX (Varian, Oxford, UK) pipette tip. The microcolumns were prepared by attaching them to a Gilson pipette, set at 100  $\mu$ L, and pipetting up and down 10 times with 80% methanol, 1% formic acid; 60% methanol, 1% formic acid; and 1% methanol, 1% formic acid. The sample (10  $\mu$ L of pronase digest) was mixed with 90  $\mu$ L of 1% methanol, 1% formic acid and applied to the microcolumn by pipetting up and down 20 times. The microcolumns were washed by pipetting up and down 20 times with 1% methanol, 1% formic acid. The pipette was reset to 50  $\mu$ L and the glycopeptides eluted by pipetting up and down 20 times with 50  $\mu$ L of 60% methanol, 1% formic acid. Aliquots of these samples were loaded into nanotips (Micromass-type F) and analyzed by ES-MS and ES-MS/MS in the positive-ion mode on an ABI Q-Star-XL instrument with tip and declustering potentials of 900 and 60 V, respectively. The product ion spectra of selected ions were collected using collision energies of 30–60 V. The ES-MS spectra were processed using the Bayesian peptide reconstruction program in the ABI Analyst software.

#### Supplementary data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org/>.

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#### Conflict of interest

None declared.

#### Abbreviations

ALG, asparagine-linked glycosylation; ER, endoplasmic reticulum; ES-MS, electrospray-mass spectrometry; GPI, glycosylphosphatidylinositol; LLO, lipid-linked oligosaccharide; MI,  $\alpha$ -mannosidase inhibitors; OST, oligosaccharyltransferase; sVSG, soluble-form VSG; TbGnTI, *T. brucei* UDP-GlcNAc: glycoprotein GlcNAc transferase type I; VSG, variant surface glycoprotein.

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