Inhibition of Nucleotide Sugar Transport in *Trypanosoma brucei* Alters Surface Glycosylation

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**Background:** Nucleotide sugar transporters (NSTs) provide availability of nucleotide sugars for glycosylation in the lumen of the Golgi apparatus.

**Results:** The substrate specificities of four *Trypanosoma brucei* NSTs were characterized, and inhibition of TbNST4 resulted in glycosylation defects.

**Conclusion:** TbNST4 plays an essential role in biosynthesis of glycoproteins in *T. brucei*.

**Significance:** To understand the roles of TbNST(s) in the growth and pathogenesis of *T. brucei*.

Nucleotide sugar transporters (NSTs) are indispensable for the biosynthesis of glycoproteins by providing the nucleotide sugars needed for glycosylation in the lumen of the Golgi apparatus. Mutations in NST genes cause human and cattle diseases and impaired cell walls of yeast and fungi. Information regarding their function in the protozoan parasite, *Trypanosoma brucei*, a causative agent of African trypanosomiasis, is unknown. Here, we characterized the substrate specificities of four NSTs, TbNST1–4, which are expressed in both the insect procyclic form (PCF) and mammalian bloodstream form (BSF) stages. TbNST1/2 transports UDP-Gal/UDP-GlcNAc, TbNST3 transports GDP-Man, and TbNST4 transports UDP-GlcNAc, UDP-GalNAc, and GDP-Man. TbNST4 is the first NST shown to transport both pyrimidine and purine nucleotide sugars and is demonstrated here to be localized at the Golgi apparatus. RNAi-mediated silencing of TbNST4 in the procyclic form caused underglycosylated surface glycoprotein EP-procyto. Similarly, defective glycosylation of the variant surface glycoprotein (VSG221) as well as the lysosomal membrane protein p67 was observed in Δtbnst4 BSF *T. brucei*. Relative infectivity analysis showed that defects in glycosylation of the surface coat resulting from *tbnst4* deletion were insufficient to impact the ability of this parasite to infect mice. Notably, the fact that inactivation of a single NST gene results in measurable defects in surface glycoproteins in different life cycle stages of the parasite highlights the essential role of NST(s) in glycosylation of *T. brucei*. Thus, results presented in this study provide a framework for conducting functional analyses of other NSTs identified in *T. brucei*.

Nucleotide sugar transporters (NSTs) play a key role in the biosynthesis of glycoproteins, as they translocate nucleotide sugars, which serve as sugar donors for glycosyltransferases in glycosylation reactions, from the cytosol into the lumen of the Golgi apparatus. Given their essential function in glycosylation, NSTs have been shown to play critical roles in development and organogenesis of mammals and higher multicellular eukaryotes as well as in the formation of cell wall or surface coat of lower unicellular eukaryotes (3, 4). Biochemical and structural studies have shown that mutations or deletions of specific NSTs resulted in defects in the particular glycoconjugates containing the sugars whose corresponding nucleotide sugar transport was compromised (3–5). More recent studies have shown that siRNA-mediated silencing of NSTs in cultured mammalian cells resulted not only in defective glycosylation of glycoproteins, but also a more global defect in the synthesis and secretion of nonglycoproteins (6). Furthermore, instances in which cell surface glycoproteins represent the main interface between a pathogen and its host, carbohydrate composition is likely to play an important role in pathogenesis.

The present study addresses the function of NSTs in African trypanosomes (*Trypanosoma brucei* ssp.), which are parasitic protozoa. These parasites cause human African trypanosomiasis, *e.g.* sleeping sickness and veterinary disease in cattle (Nagana). Infection is fatal without treatment and consequently human African trypanosomiasis represents a major health problem in sub-Saharan Africa wherever the insect vector (tsetse fly, genus *Glossina*) is found. *T. brucei* has two life cycle stages that are amenable to biochemical and biological studies:

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This article contains supplemental Table S1.

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3 The abbreviations used are: NST, nucleotide sugar transporter; PCF, procyclic form; BSF, bloodstream form; UDP-Gal, uridine diphosphate galactose; UDP-GlcNAc, uridine diphosphate N-acetylgalactosamine; UDP-GalNAc, uridine diphosphate N-acetylgalactosamine; GDP-Fuc, guanosine diphosphate fucose; GDP-Man, guanosine diphosphate mannose; SL, spiked leader; sVSG, soluble VSG; TL, tomato lectin; MAA, *Maackia amurensis* agglutinin; GSII, *Griffonia simplicifolia* II; ER, endoplasmic reticulum; ConA, concanavalin A; VSG, variant surface glycoprotein; nt, nucleotide; GPI, glycosylphosphatidylinositol.
UDP-Gal/UDP-GlcNAc, TbNST3 transports GDP-Man. TbNST4 is the first NST shown genetically and biochemically to transport both pyrimidine and purine nucleotide sugars and is demonstrated here to be localized at the Golgi apparatus. TbNST1–4 are expressed in different life cycle stages (PCF and BSF). Because of its unique substrate specificity, TbNST4 was chosen for further functional analyses. RNAi-mediated silencing of TbNST4 in PCF caused underglycosylated surface glycoprotein EP-procyclin. Similarly, defective glycosylation of VSG221 as well as the lysosomal membrane protein, p67, was observed in ∆tbnst4 BSF T. brucei. Relative infectivity analysis shows that defects in glycosylation of the surface coat resulting from tbnst4 deletion were insufficient to impact the ability of this parasite to infect mice, likely due to functional redundancy of NSTs. Overall, we demonstrate that inactivation of a single NST gene in T. brucei results in defects in glycosylation of surface proteins in different life cycle stages of the parasite, highlighting the essential role of NST(s) in glycosylation in T. brucei. Thus, results presented in this study provide a framework for conducting functional analyses of other identified TbnSTS, individually and combinatorially, to better understand their biological impact on this parasite.

**EXPERIMENTAL PROCEDURES**

**Trypanosome Growth and Transfection**

Lister 427 strain of BSF T. brucei was grown in HMI-9 medium (24) supplemented with 10% fetal bovine serum (FBS) at 37 °C in humidified 5% CO2. Lister 427 strain of PCF T. brucei was grown in SDM-79 medium (25) supplemented with 10% tetracycline-free FBS (Atlanta® Biological) at 27 °C. Logarithmic phase cells, ~1 × 10⁴/ml (BSF) and ~1 × 10⁶ (PCF), were used for conducting experiments. Plasmids used for transfection were purified using the PureYield™ Maxiprep System (Promega). The linearized DNA (10 μg) was electroporated into BSF or PCF cells using the AMAXA Nucleofector® II with program X-001 and proprietary human T-cell Nucleofector solution (Lonza, VPA-1002). Clonal cell lines were obtained by limiting dilution and selection with appropriate antibiotics.

**Total RNA Isolation and Reverse Transcription PCR**

Total RNA extraction was achieved with the RNeasy kit with on column DNase digestion (RNase-free DNase, Qiagen) or with TRIzol (Invitrogen) followed by DNase I treatment according to the manufacturer’s instructions. cDNA was obtained using the SuperScript first-strand synthesis system (Invitrogen) and RT-PCR amplification was carried out with BIO-X-ACT™ Short MiX containing DNA polymerase (Bioline). A 446-bp PCR product from nt 1 to 446 of the open reading frame was obtained for TbnST1 using TbnST1–5(F)/TbnST1–6(R) primers. A ~900-bp PCR product from nt 1 of the spliced leader to nt 600 of the open reading frame was obtained for TbnST2 using TbsLRNA-1(F)/TbnST2–2(R) primers. A ~1000-bp PCR product from nt 1 of the spliced leader to nt 781 of the open reading frame was obtained for TbnST3 using TbsLRNA-1(F)/TbnST3–6(R). A ~1220-bp PCR product from nt 1 of the spliced leader to nt 1002 was obtained for TbnST4 using TbsLRNA-1(F)/KtBnst4-B(R). Note that all trypanosome mRNAs have a 5′ spliced leader (SL)
sequence as a result of trans-splicing. All primer sequences are detailed in supplemental Table S1.

**Generation of DNA Constructs and Transgenic Trypanosome Cell Lines**

*TbNST4-RNAi PCF Cell Line*—A construct producing inducible *TbNST4* dsRNA in the form of a stem-loop structure was created as previously described in Ref. 26 using pJM325 and pLew100 vectors (gifts from Dr. Paul Englund, Johns Hopkins University). The stem sequences were from a 608-bp fragment containing the *TbNST4* coding sequence with opposite orientations. The above plasmids were linearized with EcoRV and transfected into strain 29-13 (27). Induction of *TbNST4* dsRNA was achieved with 1 μg/ml of tetracycline.

*tnbst4-null BSF Cell Line*—A *tnbst4* homozygous knock-out (KO) was created using vectors pLew13-NEO and pLew90-HYG. To generate the first allele KO construct (pSKO-*TbNST4*), the 5' and 3' UTRs of *tnbst4* were PCR amplified from BSF genomic DNA. PCR products of a 304-bp fragment containing the 5' UTR and a 327-bp fragment containing the 3' UTR were inserted into the NotI/MluI and Stul/XbaI sites of pLew13, respectively. To generate the second allele KO construct (pDKO-*TbNST4*), the SwaI/XhoI fragment containing a neomycin marker and the gene of T7 RNA polymerase was replaced with the 2491-bp StuI/XhoI fragment containing the hygromycin marker and tetracycline repressor gene from pLew90. The stable *tnbst4*-null cell line was obtained by transfection with NotI-linearized pSKO-*TbNST4* and selection with G418. Subsequently, the cell line carrying pSKO-*TbNST4* was transfected with NotI-linearized pDKO-*TbNST4* creating *tnbst4*-null mutants under the selection of 2.5 μg/ml of G418 and 5 μg/ml of hygromycin B. Introduction of T7 RNA polymerase and tetracycline genes into the genome of the double KO strain will facilitate RNAi silencing of other transporters in the future studies.

*tnbst4* in Situ Tagging in BSF Cells*—A C-terminal tagging PCR fragment was obtained using vector pMOTag3H as previously described in Ref. 28. The transgenic cell line expressing tagged *tnbst4* was generated by transfection with a 2527-bp PCR product containing the 150-bp *TbNST4* coding sequence upstream to stop codon and the 150-bp *TbNST4* 3' UTR sequence downstream to the stop codon under the selection of hygromycin B. Single marker BSF cells (29) with Ty-tagged Golgi putative glycosyltransferase (SM-BS TbGT15:15Ty) (30) was used for the cocolocalization experiment.

**Flow Cytometry**

Uninduced and induced (on day 4) PCF cells carrying the inducible *TbNST4*-RNAi construct as well as BSF WT and *tnbst4*-null mutant cells were harvested and washed with PBS. The cell pellets were resuspended at 1 × 10⁶ cells/ml in PBS and 250 μl of cell suspension aliquots were transferred to each well of a 96-well plate. Cells were incubated with mouse anti-EP, or rabbit anti-VSG221 antibodies diluted at 1:500 for the staining. Cell images were captured with a Nikon wide-field epifluorescence microscope. All images were processed using ImageJ software.

**Nucleotide Sugar Transporter in African Trypanosomes**

After washing with 0.3% BSA in PBS, cells were resuspended in 350 μl of PBS and analyzed in a flow cytometer (BD Biosciences FACScan) using detector FL1 (530 ± 30 nm). Histograms were generated with FlowJo 9.5.2 software.

**Immunofluorescence Analysis**

The procedure of immunostaining and microscope imaging for both PCF and BSF was adapted from that previously described in Ref. 31 with changes in fixation for BSF cells. Specifically, 50 μl of BSF cell suspension was pipetted onto coverslips in a 6-well plate and placed on ice for 0.5 h for the cell attachment. 4% Paraformaldehyde was used for fixation on ice for 0.5 h. Both mouse anti-EP (Cedarlane) and anti-HA (Covance) antibodies were diluted at 1:500 for the staining. Cell images were captured with a Nikon wide-field epifluorescence microscope. All images were processed using ImageJ software.

**Soluble Form VSG (sVSG) Isolation**

sVSG was isolated from 1 × 10⁶ BSF cells (in 100 ml of culture) as previously described in Refs. 20 and 32, except that the sVSG-containing fraction was not applied to an anion exchange column (DE52) but was directly applied to Western blotting.

**Antibody and Lectin Blotting**

*T. brucei* cells were lysed with RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris–HCl pH 7.5, and 150 mM NaCl) with Complete protease inhibitor mixture (Roche Applied Science). The resulting cell lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, blocked, and probed with mouse anti-EP (Covance), mouse anti-α-tubulin (Sigma), rabbit anti-EP, or rabbit anti-VSG221 antibodies. After washing, bound primary antibodies were incubated with goat anti-mouse or goat anti-rabbit IgG conjugated to horseradish peroxidase at RT for 0.5 h (Sigma). Appropriate dilutions were used for all antibodies. Lectin blotting was done as described in Ref. 20. Membranes were blocked and incubated with biotinylated ECL (5 μg/ml), RCI A (5 μg/ml), ConA (1 μg/ml), and TL (0.33 μg/ml) lectins (Vector Laboratories), with or without corresponding sugar inhibitors. Bound biotinylated lectins were detected with streptavidin-peroxidase (Kirkegaard & Perry Laboratory). All membranes were developed after treatment with ECL chemiluminescent reagent (GE Healthcare).

**Metabolic Radiolabeling and Immunoprecipitation**

Pulse-chase metabolic radiolabeling and subsequent immunoprecipitation of radiolabeled proteins from cell lysates were performed as previously described (33, 34). Briefly, BSF WT (VSG221) and *tnbst4*-null cells were pulse labeled with 200 μCi of [³⁵S]Met/Cys for 15 min and then chased at 1:10 dilution with complete HML-9 for 2 h in the presence of FMK024 (20 μM) (MP Biomedicals, Aurora, OH), a selective thiol protease inhibitor. Immunoprecipitated proteins were analyzed by 10% SDS-PAGE and fluorography.

**Mouse Infection**

Parental BSF *T. brucei* (VSG221) and *tnbst4*-null cells were grown to a density of 1 × 10⁶ without selection drugs (hygro-
**Nucleotide Sugar Transporter in African Trypanosomes**

mycin B and G418) for 24 h. C57BL6 mice (5 per group) were infected intraperitoneally with 10^5 or 10^6 parasites in 0.2 ml of HMI-9 medium. Relative infectivity was assessed by determining parasitemia in tail vein blood using a Neubauer hemocytometer.

**Yeast Strain Maintenance and Transfection**

Saccharomyces cerevisiae strain, PRY225 (ura3–52 lys2–801am ade2–1020 his3 leu2 trplΔI), and Kluyveromyces lactis mutant strain, KL3 (MATα, uraA, mnn2–2, argK+, pKD1+) (35) and its isogenic parental strain, KL8 (MG1/2, MATα, uraA, arg− lys− K+, pKD1+), were grown at 30 °C in liquid culture consisting of 1% yeast extract, 2% Bacto-peptone, 2% dextrose (YPD) or on solid YPD medium containing 2% Bacto-agar (36).

*S. cerevisiae* mutant strain, NDY5 (vrg4–2) (MATα, ura3–52, leu2–211, vrg4–2) (37), and its isogenic parental strain, RSY255 (MATα, ura3–52, leu2–211), were grown at the same conditions described above except that an additional 0.004% adenine sulfate was added. Transformation of *S. cerevisiae* and *K. lactis* was carried out using the lithium acetate method (38). Briefly, yeast competent cells were mixed with salmon sperm carrier DNA, 1 μg of plasmid, and polyethylene glycol (PEG) mixture (5% PEG, 0.1 μl lithium acetate in 1X TE buffer) were incubated at 30 °C for 30 min and heat shocked at 42 °C for 5 min before plating.

**Generation of DNA Constructs Expressing TbNSTs in *S. cerevisiae* and *K. lactis***

To generate the constructs expressing TbNSTs in *S. cerevisiae*, the open reading frame sequences of the TbNSTs were PCR amplified from PCF genomic DNA. The 1113-, 1359-, and 1056-, and 1002-bp of PCR products were inserted into vector p426GPD at the BamHI/EcoRI sites creating p426GPD-TbNST1 and p426GPD-TbNST3, at the SpeI/XhoI sites creating p426GPD at the BamHI/EcoRI sites creating p426GPD and p426GPD-TbNST2, and at the BglII/XhoI sites creating TbNST1 and p426GPD-TbNST4, at the SpeI/XhoI sites creating p426GPD-TbNST2, and at the BglII/XhoI sites creating p426GPD-TbNST1–4, each individually, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Transformants were grown for 72 h and thereafter seeded on coverslips. After 24 h of incubation, washed cells were fixed with 2% paraformaldehyde. After washing, cells were blocked with 3% BSA in PBS at RT for 1 h and incubated with MAA-FITC (40 μg/ml) (EY Laboratories) at RT for 1 h, followed by fluorescence microscopy.

**Congo Red Assay—*S. cerevisiae* WT and vrg4–2 mutant (defective in GDP-Man transport) cells (37), transfected with empty vector p426GPD and p426GPD-TbNST1–4, each individually, were grown to mid-logarithmic phase in the synthetic complete medium minus uracil, diluted to absorbance of ~0.2 at 600 nm, and equal volumes were applied onto uracil minus plates containing 0–50 μg of Congo red. Plates were scored and photographed after 3 days at 30 °C.

**RESULTS**

**Identification of T. brucei Nucleotide Sugar Transporters—**

To identify TbNSTs, we used BLASTp to search the *T. brucei* predicted protein database (tritrypdb.org) for homologs with well characterized NSTs from different organisms such as *L. major*, *C. elegans*, and *Homo sapiens*. Eight proteins were revealed to be putative NSTs, referred to as TbNST1–8. The evolutionary distances of the amino acid divergence between or among the putative TbNSTs and known NSTs from different organisms are depicted by a phylogenetic tree (Fig. 1). TbNST1 and TbNST2 share the most conserved amino acid identity with known UDP-sugar transporters such as for UDP-Gal and UDP-GlcNAc (Fig. 2), whereas they show little sequence relationship with GDP-sugar transporters. TbNST3 and TbNST4 share the most conserved amino acid identity with known GDP-Man transporters and also show the similarity to the GDP-Fuc transporters (Fig. 3). Of the eight candidate NSTs, TbNST1–4 were selected for further experimental evaluation, based on their high degree of amino acid sequence homology with known transporters.

It is known that protein expression in *T. brucei* during the different life cycle stages is highly dynamic and stage specific. To examine the expression of TbNST1–4, total RNA from both PCF and BSF cells was isolated, reverse transcribed, and...
the mRNA transcripts were examined. Fig. 4 shows that TbNST1–4 are all expressed in PCF (left panel) and BSF (right panel) T. brucei.

Substrate Specificity of TbNST1–4—Previous studies from our and other laboratories showed that the substrate specificity of NSTs cannot be simply predicted from primary sequence comparisons (1, 2). NSTs sharing 50–60% of amino acid sequence identity may have different substrate specificities, whereas NSTs sharing as little as 20% of amino acid sequence identity may have the same substrate specificity (3, 5). Therefore, the substrate specificity of putative TbNSTs must be determined experimentally. To this end, we have used two approaches: (a) in vitro biochemical transport assays using yeast S. cerevisiae Golgi-enriched vesicles expressing the tagged TbNSTs and (b) in vivo genetic complementation of previously well characterized yeast or mammalian mutants, specifically defective in transport of the particular nucleotide sugars.

S. cerevisiae has been a powerful system to determine the substrate specificity of putative NSTs, because it contains only two endogenous nucleotide sugar transport activities, those for UDP-Glc and GDP-Man. To evaluate the transport activities of putative TbNSTs for nucleotide sugars except the above mentioned two endogenous substrates, we transformed WT S. cerevisiae cells (Pry225) with empty vector p426GDP or p426GDP containing TbNST1–4–VSV separately. The Golgi-enriched vesicles expressing TbNSTs were isolated and assayed for transport of radiolabeled UDP-Gal, UDP-GlcNAc, UDP-GlcNAc, and GDP-Fuc as previously described (39). As shown in Fig. 5, A and B, the transport activity of vesicles expressing TbNST1 and TbNST2 for UDP-Gal and UDP-GlcNAc is at least 2-fold higher than that of the empty vector; no transport activity for GDP-Fuc or UDP-GalNAc was detected. Transport activity of vesicles expressing TbNST4 for UDP-GlcNAc and UDP-GalNAc is ~3.5–4-fold higher than that of the empty vector; minor or no transport activity was detected for UDP-Gal or GDP-Fuc, respectively (Fig. 5D).

Notably, one of the characteristics of NSTs is their rate-limiting transport of substrates with an apparent K_m in the 1–30 μM range. As shown in Fig. 5E, transport of UDP-GlcNAc by TbNST4 was saturable with a K_m of 14.0 μM. No transport activity for UDP-Gal, UDP-GlcNAc, UDP-GalNAc, or GDP-Fuc was detected for TbNST3 (Fig. 5C).

To further evaluate the UDP-GlcNAc substrate specificity for TbNST1, -2, and -4, we used a K. lactis mutant strain, KL3, which was previously shown to be specifically defective in UDP-GlcNAc transport (35). This mutant lacks terminal GlcNAc in its glycoproteins at the cell surface and therefore, has a surface binding defect to the GSII lectin, which recognizes terminal α- or β-linked GlcNAc. Thus, TbNST1–4 were individually expressed in the KL3 mutant strain and phenotypic correction was measured by restoration of cell surface binding of fluorescently labeled GSII lectin. As shown in Fig. 6A, expression of TbNST4 restored GSII binding to the cell surface of KL3 mutant cells to amounts comparable with that of WT KL8 cells; a modest restoration was also detected for TbNST1–3 as compared with that of KL3 transformants with the empty vector.

To further determine whether TbNST1 and TbNST2 transport UDP-Gal, we used a mutant Madin-Darby canine kidney cell line, RCar, which was previously shown to be specifically defective in UDP-Gal transport (41). Lack of galactose in glycoproteins at the cell surface renders this mutant strain defective in UDP-Gal transport (41). Lack of galactose in glycoproteins at the cell surface renders this mutant strain defective in UDP-Gal transport (41). Lack of galactose in glycoproteins at the cell surface renders this mutant strain defective in UDP-Gal transport (41). Lack of galactose in glycoproteins at the cell surface renders this mutant strain defective in UDP-Gal transport (41). Lack of galactose in glycoproteins at the cell surface renders this mutant strain defective in UDP-Gal transport (41).

Based on sequence comparisons, TbNST3 and TbNST4 most likely transport GDP-Man. It is known that WT S. cerevisiae has a high endogenous GDP-Man transport activity. To test whether these two transporters transport GDP-Man, we used a mutant S. cerevisiae strain, vrg4–2, which was previously shown to have a significant defect in transport of GDP-Man (37, 42), therefore, resulting in much lower endogenous GDP-Man transport activity than WT. Thus, vrg4–2 mutant cells were transfected with empty vector p426GDP or p426GDP-TbNST1–4 individually. Following isolation of Golgi-enriched vesicles expressing the TbNSTs, transport assays were performed as described for WT S. cerevisiae. Fig. 6C shows that expression of TbNST3 or TbNST4 increased transport activity of vrg4–2 mutant cells by ~5- or ~3-fold, respectively, as compared with that of vrg4–2 transformed with the empty vector. No restoration of GDP-Man transport activity for vrg4–2 mutant cells was detected for TbNST1 or TbNST2 (data not shown).
shown). To further validate the above in vitro results, we performed an in vivo genetic complementation experiment using the same \(vrg4^{-2}\) mutant. The cell wall of \(vrg4^{-2}\) mutants is known to be profoundly compromised due to a lack of surface mannoproteins (43), which is compensated by an increase of cell surface chitin. This results in hypersensitivity of \(vrg4^{-2}\) mutant cells to Congo red, an anionic dye that binds chitin and kills cells by disrupting cell wall formation. \(Vrg4^{-2}\) mutant cells expressing \(TbNST1–4\), respectively, and transformants with the empty vector were grown on YPDA agar plates in the presence of different concentrations of Congo red. As shown in Fig. 6D, both TbNST3 and TbNST4 restored Congo red resistance of \(vrg4^{-2}\) mutant cells, whereas TbNST1 and -2 did not (data not shown).

Thus, based on these collective findings, we conclude that TbNST1 and TbNST2 transport UDP-Gal and UDP-GlcNAc, TbNST3 transports GDP-Man, and TbNST4 transports UDP-GlcNAc, UDP-GalNac, and GDP-Man as summarized in Table 1. To our knowledge, this is the first NST characterized biochemically and genetically to transport both pyrimidine and purine nucleotide sugars.

**TbNST4 Is Localized at the Golgi Apparatus in \(T. brucei\)**—In other eukaryotes, most nucleotide sugars are synthesized in the cytosol, and translocated by NSTs into the lumen of the Golgi apparatus. In \(T. brucei\), synthesis of nucleotide sugars takes place in glycosomes (44–46). This raises the question of whether these NSTs identified so far reside in membranes of glycosomes, the Golgi apparatus, or both. To determine the subcellular localization of TbNSTs, we used one-step PCR in situ tagging methodology to tag the endogenous \(tbnst4\) with 3×/H11003 HA coding sequence at its C terminus (28). This method minimizes the likelihood of mislocalization of tagged proteins from overexpression. A 1842-bp PCR product containing a tagging cassette, which is specific to target the C-terminal locus of the \(tbnst4\) gene, was transfected into the single marker BSF strain (29) with Ty-tagged Golgi putative glycosyltransferase (SM-BS TbGT15:Ty) (30). Following the establishment of a stable transgenic cell line, the in situ tagging locus was confirmed by genomic PCR. 

**FIGURE 2. Amino acid sequence alignment of the \(T. brucei\) TbNST1 and TbNST2 with UDP-sugar transporters from other organisms.** Sequence alignment was carried out using the ClustalW program. Identical amino acids are highlighted in red and conserved substitutions are in blue. TbNST1 (Tb927.10.13900) and TbNST2 (Tb927.11.16560), putative NSTs, from \(T. brucei\); EhNST1 (EAL43205), a UDP-Gal transporter, from *Entamoeba histolytica*; SpUGT (NP_58804), a UDP-Gal transporter, from *Schizosaccharomyces pombe*; CeC03H5.2 (AAB66127), a UDP-GalNAc/UDP-GlcNAc transporter, from *C. elegans*; and HsUGT (N_P005651), a UDP-Gal transporter, from *H. sapiens*. 

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**Table 1.**

| Sugar Type | Gene |
|------------|------|
| UDP-Gal    | TbNST1 |
| UDP-GlcNAc | TbNST2 |
| GDP-Man    | TbNST3 |
| UDP-GlcNAc | TbNST4 |

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**FIGURE 2.** Amino acid sequence alignment of the \(T. brucei\) TbNST1 and TbNST2 with UDP-sugar transporters from other organisms. Sequence alignment was carried out using the ClustalW program. Identical amino acids are highlighted in red and conserved substitutions are in blue. TbNST1 (Tb927.10.13900) and TbNST2 (Tb927.11.16560), putative NSTs, from \(T. brucei\); EhNST1 (EAL43205), a UDP-Gal transporter, from *Entamoeba histolytica*; SpUGT (NP_58804), a UDP-Gal transporter, from *Schizosaccharomyces pombe*; CeC03H5.2 (AAB66127), a UDP-GalNAc/UDP-GlcNAc transporter, from *C. elegans*; and HsUGT (N_P005651), a UDP-Gal transporter, from *H. sapiens*. 

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**Figure 7A** shows the genomic PCR products for both the untagged and tagged loci (Fig. 7A, lanes 1 and 2) and as expected, a 2527-bp fragment was detected only in cells expressing the tagged gene (Fig. 7A, lane 2, but not lane 1). Expression of the tagged TbNST4 protein was confirmed by Western blotting with anti-HA antibody (Fig. 7B, lane 2). The localization of the tagged TbNST4 in BSF cells was visualized by immunofluorescence. As shown in Fig. 7C, HA-tagged TbNST4 (green) was confined to a single dot in the postnuclear region in close association with the flagellar attachment zone, as is typical for the Golgi in trypanosomes (47), rather than multiple glycosomal microbodies. It colocalized with...
Ty-tagged Golgi marker, glycosyltransferase (red, upper panel), but not with p67, a lysosomal membrane protein (red, lower panel). The above results therefore strongly suggest that TbNST4 is localized at the Golgi apparatus.

RNAi-mediated Silencing of TbNST4 in PCF T. brucei and Its Effect on Cell Growth—To investigate the role of TbNST4 in PCF cells, we generated a conditional RNAi cell line carrying the construct to produce the stem-loop dsRNA specific for TbNST4 mRNA. Gene expression of TbNST4 can be knocked down by induction with tetracycline (26). To examine the efficiency and specificity of RNAi silencing, total RNA was isolated from uninduced and induced cells (on day 4), and RT-PCR was performed for detection of RNA transcripts of TbNST4 and control genes. As shown in Fig. 8A, the mRNA level of TbNST4 in induced cells was reduced by above 90% (lane 3) as compared with that of WT and uninduced cells (lanes 1 and 2). This reduction was TbNST4-specific, because mRNA levels of TbNST2 (Fig. 8A, right panel, lane 3) and TbNST3 (Fig. 8A, left panel, lane 3) were not affected.

Silencing of TbNST4 causes a modest growth defect (Fig. 8B); the doubling time of induced cells was delayed by 3 h as compared with that of uninduced cells (21 versus 18 h). After induction of TbNST4 dsRNA for 4 days, no apparent morphological changes were visualized for TbNST4 silenced cells via light microscope (data not shown).

TbNST4 Is Essential for the Maturation of the Major Surface Glycoprotein, EP-Procyclin—To investigate the effect of TbNST4 silencing on the glycosylation in PCF cells, we examined the maturation of EP-procyclins, one of the major surface GPI-anchored glycoproteins, which is characterized by an internal dipeptide, /H1/10/20 of glutamate-proline (EP) repeat. Two of the three EP procyclins have a single high mannose N-glycan that is not pro-

Ty-tagged Golgi marker, glycosyltransferase (red, upper panel), but not with p67, a lysosomal membrane protein (red, lower panel). The above results therefore strongly suggest that TbNST4 is localized at the Golgi apparatus.

RNAi-mediated Silencing of TbNST4 in PCF T. brucei and Its Effect on Cell Growth—To investigate the role of TbNST4 in PCF cells, we generated a conditional RNAi cell line carrying the construct to produce the stem-loop dsRNA specific for TbNST4 mRNA. Gene expression of TbNST4 can be knocked down by induction with tetracycline (26). To examine the efficiency and specificity of RNAi silencing, total RNA was isolated from uninduced and induced cells (on day 4), and RT-PCR was performed for detection of RNA transcripts of TbNST4 and control genes. As shown in Fig. 8A, the mRNA level of TbNST4 in induced cells was reduced by above 90% (lane 3) as compared with that of WT and uninduced cells (lanes 1 and 2). This reduction was TbNST4-specific, because mRNA levels of TbNST2 (Fig. 8A, right panel, lane 3) and TbNST3 (Fig. 8A, left panel, lane 3) were not affected.

Silencing of TbNST4 causes a modest growth defect (Fig. 8B); the doubling time of induced cells was delayed by 3 h as compared with that of uninduced cells (21 versus 18 h). After induction of TbNST4 dsRNA for 4 days, no apparent morphological changes were visualized for TbNST4 silenced cells via light microscope (data not shown).

TbNST4 Is Essential for the Maturation of the Major Surface Glycoprotein, EP-Procyclin—To investigate the effect of TbNST4 silencing on the glycosylation in PCF cells, we examined the maturation of EP-procyclins, one of the major surface GPI-anchored glycoproteins, which is characterized by an internal dipeptide, ~20–30 of glutamate-proline (EP) repeat. Two of the three EP procyclins have a single high mannose N-glycan that is not pro-
cessed in the Golgi apparatus (7, 13, 48). However, GPI anchors of procyclins are heavily modified with the large branched poly-LacNAc (Gal\(\beta\)1–4GlcNAc)-containing side chains with an average of about 8–12 repeats (49). EP-procyclins from total cell lysates of uninduced and induced cells (for 4 days) were subjected to Western blotting and detected with anti-EP antibody. The results showed that EP-procyclins from TbNST4 silenced cells migrated faster than that of WT cells (Fig. 9A, comparison of lane 2, Tet\(^+)/H\) with lane 1, Tet\(^–/H\)) indicating a reduction in the apparent molecular weights of EP-procyclins. This strongly suggests that silencing TbNST4 gene expression causes underglycosylated EP-procyclins, whereas there is no apparent decrease in total EP.

We next asked whether underglycosylated EP-procyclin affects its transport to the cell surface. If this is the case, a reduction of surface EP-procyclins would be expected in TbNST4-silenced cells. To examine surface EP-procyclins, live cells uninduced and induced on day 4 after addition of tetracycline were incubated with anti-EP antibody and subjected to flow cytometric analysis. Fig. 9B shows that EP-antibody surface binding to induced cells (reflected by the intensity of fluorescence) was reduced by 48% as compared with that of uninduced cells suggesting a transport defect.

To further examine the distribution and localization of EP-procyclins at the surface and within TbNST4-silenced cells, immunofluorescence analyses were performed with anti-EP antibody with
or without cell permeabilization. As shown in Fig. 9C (left panel), in uninduced, permeabilized cells, most EP-procyclins were on the cell surface, whereas in induced cells, significantly less EP-procyclins were seen on the surface, with a compensatory increase in internal staining in a location consistent with the Golgi apparatus in PCF cells (47). In Fig. 9C (right panel), in uninduced, nonpermeabilized cells, EP-procyclins were homogenously distributed at the cell surface, whereas considerably less staining could be seen on the surface of the induced cells. These results are in good agreement with the above flow cytometric analysis. Overall, these results strongly suggest that silencing of TbNST4 results in a glycosylation defect of EP-procyclin and further affects its translocation to the cell surface.

**Knock-out of TbNST4 Gene in BSF T. brucei and Its Effect on Cell Growth—** As shown in Fig. 4, TbNST4 is expressed in both PCF and BSF life cycle stages of *T. brucei*. Because BSF *T. brucei* infects mammals, we decided to examine the role of TbNST4 in growth, glycosylation, and infectivity. As described above, *T. brucei* is a diploid; we knocked out two alleles of the TbNST4 gene to generate a double KO (dKO) cell line. The TbNST4 gene to generate a single KO (sKO) cell line. The second step transfection introduced a gene expressing a hygromycin selection marker (HYG) into the locus of the one allele of the TbNST4 gene by successive two-step transfections. The first step transfection introduced a gene expressing a neomycin selection marker (NEO) into the locus of the other allele of the TbNST4 gene to generate a single KO (sKO) cell line. The second step transfection introduced a gene expressing a hygromycin selection marker (HYG) into the locus of the other allele of the TbNST4 gene to generate a double KO (dKO) cell line. The *tbnst4*-null mutants (*Δtbnst4-neo/Δtbnst4-hyg*) were selected in the presence of neomycin and hygromycin B.

Replacement of the two alleles was confirmed by genomic PCR amplification of the *tbnst4* locus using two pairs of primers: one pair with a forward primer complementary to 5′ UTR outside the recombination sequence and a reverse primer com-
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FIGURE 7. TbNST4 is localized at the Golgi apparatus. A, genomic PCR analysis of in situ C-terminal HA-tagged tbnst4 in T. brucei BSF. Genomic DNA was isolated from untagged (lane 1) and tagged (lane 2) TbNST4 cells. In situ tagging locus was PCR amplified with a forward primer complementary to TbNST4 coding sequence and a reverse primer complementary to the 3′ UTR (outside the recombination sequence). The expected genomic PCR products are indicated. B, Western blot analysis of the in situ tagged TbNST4HA protein expression. Cell lysates from untagged (lane 1) and tagged TbNST4HA (lane 2) cells were subjected to SDS-PAGE and Western blotting with anti-HA antibody detection. The position of the expressed TbNST4 is indicated. C, immunofluorescence analysis of TbNST4HA localization. BSF cells carrying the tagged TbNST4 were stained with anti-HA for TbNST4 (green), anti-Ty for TbgT15 (red, upper panel), or anti-p67 (red, lower panel) antibodies. Cell images were captured with Nikon wide-field epifluorescence microscope. Blue, DAPI staining for nuclei and kinetoplasts.

TbNST4 is important, but not essential for BSF T. brucei growth.

Knock-out of the TbNST4 Gene Causes Glycosylation Defects in BSF T. brucei—Earlier biochemical studies showed that glycoconjugates were deficient in the particular sugar as well as those sugars linked to the particular sugar for which the corresponding nucleotide sugar transport was defective (3, 5). We therefore expected defects in glycoconjugates containing GlcNAc and mannose in a tbnst4-null mutant, as TbNST4 transports UDP-GlcNAc and GDP-Man. To assess the glycosylation phenotype in tbnst4-dKO cells, we took advantage of the commercial availability of a variety of biotinylated or fluorescently labeled lectins, which recognize the above two sugar-containing carbohydrates. Those include (a) ECL lectin, which recognizes a single LacNAc, (b) ConA lectin, which recognizes α-linked mannose, (c) RCA I lectin, which recognizes the terminal galactose preferentially linked to GlcNAc, and (d) TL, which recognizes poly-LacNAc. We quantitatively measured the surface binding by flow cytometry of the above four fluorescent lectins to WT and two tbnst4-dKO clonal cell lines. As shown in Fig. 11, surface lectin binding to tbnst4-dKO cells (reflected by the intensity of fluorescence), compared with WT, was reduced by ~52 (ECL, Fig. 11A), ~49 (RCA1, Fig. 11B), and ~55% (TL, Fig. 11C). Interestingly, the ConA lectin binding of two tbnst4-dKO clonal cell lines slightly was increased as compared with that of WT (Fig. 11D) suggesting perhaps, a compensatory effect. Because the three lectins, which show a binding defect in tbnst4-dKO cells, share LacNAc (either single or
predicted, TL did not bind to sVSG, as it lacks poly-LacNAc; this serves as a negative control (Fig. 13B). A slight decrease in binding was detected with TL for sVSG-depleted cell lysates in tbnst4-dKO cells (Fig. 13D, left panel). Again, the ability of appropriate sugars to compete for lectin binding on blots demonstrates specificity (Fig. 13, A, C, and D, right panels). All these biochemical data are consistent with the surface lectin binding results obtained from flow cytometry. Importantly, similar lectin binding results were also obtained from RNAi-mediated TbnSt4 knockdown of BSF cells (data not shown) suggesting phenotypic glycosylation specificity. In summary, KO of tbnst4 causes a defect in LacNAc-N-linked glycoproteins and had no apparent effect on high mannose oligosaccharides.

It was important to assess the effect of tbnst4 KO on glycosylation of other proteins in BSF cells; therefore, we examined the synthesis and maturation of p67 and the cathepsin L orthologue (TbCatL) (51, 52). Both are well characterized lysosomal glycoproteins. p67 is synthesized as a 100-kDa glycoform in the ER and further modified with poly-LacNAc in the Golgi apparatus resulting in a 150-kDa glycoform (34), whereas TbCatL is synthesized in the ER as a proprotein with 2 oligomannose N-glycans without further processing during transport to the lysosome (53). Because p67 contains the poly-LacNAc structure, we speculated, based on the above lectin binding results, that synthesis of p67 would be affected in the tbnst4-dKO cells. Nascent polypeptides from WT or dKO cells were pulse-labeled with 35S for 15 min and chased for 2 h. EMK024, a lysosomal cysteine protease inhibitor (54), was used to block turnover of p67 and TbCatL. The extracts were immunoprecipitated with anti-p67 or anti-TbCatL antibodies, respectively. Mature p67 from tbnst4-dKO cells exhibited a faster mobility as compared with that of WT (Fig. 14, comparison of lane 4 with lane 2 in top panel). No difference in mobility between WT and dKO cells was detected for TbCatL (Fig. 14, bottom panel). These results are consistent with the above conclusion, namely, KO of tbnst4 results in a defect in synthesis of LacNAc and has no effect on the synthesis of high mannose oligosaccharides.

**The Effect of tbnst4 Knock-out in BSF T. brucei on Growth and Infectivity in Mice—**To determine whether loss of tbnst4 expression and the concomitant effect on the glycosylation of VSG impacts the ability of T. brucei BSF to survive in the mammalian bloodstream, 8-week-old C587BL6 mice (five in each group) were inoculated intraperitoneally with 102 or 103 WT or tbnst4-dKO parasites and parasitemia were monitored daily. Lethal parasitemias (~109/ml) were reached within 5 days with an inoculum of 103 WT (Fig. 15A, right panel) and 6 days for 102 parasites (Fig. 15A, left panel). With an inoculum of 102 WT parasites, all mice died before day 6 post-infection (Fig. 15B); however, with the same inocula of tbnst4-dKO parasites, a clearance of circulating parasites occurred in 2 out of 4 mice between days 5 and 7 with a relapse afterward (Fig. 15C). Thus, tbnst4 deletion in T. brucei resulted in a slight delay in the onset of parasitemia and mortality, possibly related to the slight decrease in the doubling time of these mutants (data not shown), however, overall virulence was not affected.
DISCUSSION

As a first step toward understanding the roles of TbNSTs in glycosylation of surface glycoconjugates and *T. brucei* infectivity, we identified eight putative TbNST1–8 from the predicted protein database by comparison of sequence homology with known NSTs. The substrate specificities of TbNST1–4 were experimentally determined using *in vitro* biochemical transport assays and *in vivo* genetic complementation of previously well defined yeast and mammalian mutants, defective in the transport of particular nucleotide sugars as summarized in Table 1. *T. brucei* is one of the earliest diverging unicellular organisms in eukaryotic evolution; many of the biochemical features of its glycosylation pathways differ from those in its mammalian hosts (49, 55). These features include: (a) the modification of most of its membrane and secretory proteins with N-linked glycans (56), whereas O-linked GalNAc, the major type of O-linked glycans of mammalian cells, has not been reported in *T. brucei*. No UDP-GalNAc was found in...
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In this study, we characterized two GDP-Man transporters in T. brucei. TbNST4 was localized to the Golgi apparatus. This motivated us to determine the location of TbNST3, which is evolutionarily related to peroxisomes in higher eukaryotes and their function is essential in carbohydrate metabolism in T. brucei. However, in its mammalian hosts, most nucleotide sugars are synthesized in the cytosol. T. brucei nucleotide sugars following their synthesis in glycosomes must be translocated into the cytosol via unidentified membrane transporters. This motivated us to determine the location of TbNST4. By analogy to most known NSTs in eukaryotes, TbNST4 was localized to the Golgi apparatus. In this study, we characterized two GDP-Man transporters in T. brucei. GDP-Man transporters have been identified in a variety of lower eukaryotes such as Leishmania, yeast, and fungi and have been shown to be essential for virulence of some Leishmania strains. Mammals, however, do not use GDP-Man transport and lack a GDP-Man transporter gene. Instead, they exclusively use a lipid donor, dolichol-phosphate-mannose, which is translocated into the ER by lipid flipping. The possibility that T. brucei has glycoconjugates with mannose being added in the Golgi apparatus cannot be ruled out. Studies of T. brucei KO biochemical and biological phenotypes may shed light on this issue. The differences in the requirement for Golgi GDP-Man transport between the lower eukaryotic pathogens and their mammalian hosts makes this transporter a potential target for possible chemotherapeutic intervention.

Although T. brucei exhibits unique characteristics in glycosylation pathways, the identified TbNSTs are conserved in many aspects with known NSTs from other organisms, e.g. their primary amino acid sequence, molecular weight, topology of the predicted transmembrane domains, and biochemical transport characteristics such as $K_m$ values in the lower micromolar range. Specifically, TbNST3 and TbNST4 contain a consensus motif "VGXLNK" shared among GDP-Man transporters of Leishmania Lpg2p, fungi Gmt1, and yeast Vrg4p, which has been shown to be involved in affinity and transport in S. cerevisiae.

Unique to TbNST4 is its capacity to transport both pyrimidine and purine nucleotide sugars (UDP-GlcNAc, UDP-GalNAc, and GDP-Man). In previous studies, NSTs were found to transport either pyrimidine or purine nucleotide sugars...
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exclusively. Although a few previous studies suggested that specific NSTs transport both types of substrates, the evidence was not compelling as only a biochemical approach was used with signals below twice the background levels and no genetic complementation analyses were performed (70). The fact that TbNST4 transports both types of nucleotide sugars and also transports UDP-GalNAc, a nucleotide sugar, which has not been reported to date in T. brucei and this organism lacks GalNAc in its glycoconjugates, suggests that the recognition and affinity of NSTs for their nucleotide sugar substrates may be less strict than previously found in other organisms.

The important roles of TbNST4 in two major surface glycoproteins, EP-procyclin in PCF and VSG221 in BSF cells, have been demonstrated by specific silencing in PCF and gene disruption in BSF cells, respectively. TbNST4-silenced PCF cells expressed underglycosylated surface EP-procyclins with the lower apparent molecular weights (faster mobilities on SDS-PAGE). We hypothesize that this defect results from impaired synthesis of LacNAc-containing GPI anchor side chains rather than N-glycosylation defects. This is based on the following findings. (a) The procyclic GPI anchor precursors are assembled in the ER and further modified with large heterogeneous glycan side chains by addition of poly-LacNAc structures in the Golgi apparatus (12, 15), where TbNST4 resides. (b) Two of the three EP-procyclins have only a single high mannose N-glycan, which is added to the nascent proteins in the ER and is not further processed in the Golgi apparatus. (c) A similar smaller size pattern of procyclins was also observed in null mutants of tbgt8 (encoding a UDP-GlcNAc:β-Gal-GPIβ1–3GlcNAc transferase) as a result of changes in the procyclin GPI anchor side chains (23).

Silencing of TbNST4 causes a reduction of EP-procyclins at the cell surface suggesting a defect in transport, which may also be the result of an impaired GPI anchor structure. The GPI

FIGURE 14. KO of tbnst4 causes the glycosylation defect of the lysosomal membrane protein, p67. WT and dKO cells were pulse-labeled with 35S for 15 min and chased for 2 h in the presence of FMK024 (a lysosomal thiol protease inhibitor). Extracts prepared at time 0 (lanes 1 and 3) and 2 h (lanes 2 and 4) were used for immunoprecipitation with antibodies against p67 (top) or TbCatL (bottom). Precipitation products were separated by SDS-PAGE and visualized by fluorography. The multiple migration bands of the gp150 glycoform of p67 from WT and dKO cells are indicated. TbCatL serves as a negative control. P, proprotein. X, an unknown product at time 0.

FIGURE 15. KO of tbnst4 causes a slight delay in parasitemia and mortality in mice. – 8-Week-old female C587BL6 mice (4 – 5/group) were inoculated intraperitoneally with 10^2 or 10^3 parasites. Parasitemia was measured by blood parasite numbers counted with a hemacytometer and (A) averaged at the indicated days or (B) plotted for individual mice for WT 10^2 inoculum and (C) for dKO 10^2 inoculum. Dpi, days post-infection.
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anchor has been shown to play an important role in forward secretory trafficking (71). Although significant glycosylation defects of EP-procytins were observed in the TbNST4-silenced cells, this had little impact on cell viability. These results are consistent with previous studies showing that depletion of procytins on the cell surface, either by impairing GPI anchor synthesis or KO of total procytin genes (gpteet and epi–3), had little or no effect on cell growth. Further studies revealed that the absence of cell surface procytin is compensated by an increase of glycosylinositophospholipids (72–74). Importantly, parasites lacking procytins on their surface were unable to infect their insect vector, tsetse flies (73, 75). Based on these studies, we speculate that PCF tbnst4-dKO cells might be impaired in their ability to infect tsetse flies.

Likewise deletion of tbnst4 in BSF cells also caused glycosylation defects in synthesis of LacNAc-containing proteins. This was demonstrated by a decrease in binding of ECL and TL lectins to live cells by flow cytometry (Fig. 11, A and C) and in binding of ECL to sVSG-depleted lysate proteins by lectin blots (Fig. 12C). We expected a glycosylation defect in VSG221 and p67 in tbnst4-dKO cells, because both are modified with complex glycans, which contain either single or poly-LacNAc structures (19, 34, 76). Indeed, a reduction in the amount of ECL lectin binding was observed for sVSG221 (Fig. 12A) and an impaired biosynthesis of mature glycoprotein was detected for p67 (Fig. 14). However, no changes in ConA binding to either sVSG or sVSG-depleted total cell lysates were detected in tbnst4-dKO cells (Fig. 12, B and D) suggesting that the oligomannose N-glycans were not affected by KO of tbnst4. Consistent with these results, synthesis and maturation of TbCatL, which is modified only by two oligomannose N-glycans, was not affected by KO of tbnst4. This is most likely because addition of oligomannose N-glycans to nascent proteins in T. brucei occurs in the ER. However, we cannot completely rule out that the GDP-Man transport activity reflects a relaxed substrate specificity by TbNST4 and TbNST3 transporters.

In PCF cells, silencing of TbNST4 causes a drastic reduction in the EP-procytin molecular weight, whereas, in BSF tbnst4-dKO cells, changes in the apparent molecular weight of VSG221 were not observed. This could be explained by the major differences of processing of GPI anchors in these two different life cycle stages. In PCF cells, GPI anchors of EP-procytins are extensively modified by large branched poly-LacNAc side chains (12, 21). A reduction in EP-procytin molecular weights indicates a significant decrease in total poly-LacNAc synthesis, whereas in BSF cells, the GPI cores are only added by several terminal or branched galactoses (up to 8) (15, 22). KO of tbnst4 in BSF cells appears to only affect the complex sugar structure on one of the two N-glycans, which may not be sufficient to cause a change in electrofrophoretic mobility, whereas showing a reduction in binding of ECL lectin.

Even though KO of tbnst4 resulted in underglycosylated surface VSGs, no major effect on cell growth or parasite infectivity was observed. We speculate that this may be the result of functional redundancy of NSTs. Several lines of evidence support this hypothesis. (a) Several TbNSTs transport the same nucleotide sugars, e.g. TbNST1, -2, and -4, all transport UDP-GlcNAc and both TbNST3 and TbNST4 transport GDP-Man. Additionally, substrates for the other four putative TbNSTs remain to be identified. (b) Functional redundancy has previously been observed for C. elegans NSTs, e.g. srf-3, a UDP-Gal/UDP-GlcNAc transporter mutant alone did not show any morphological change, but silencing of another NST, C03H5.2 (which transports UDP-GlcNAc/UDP-GalNAc) in srf-3 mutant cells, caused major developmental defects (40, 77). (c) We found that in mice inoculated with tbnst4-KO cells, parasitemia was attenuated in the early stage of infection, probably due to a slower cell growth, but thereafter parasites were able to survive, replicate, and induce disease normally as WT. This suggests that other alternate pathways, leading to compensate the function of TbNST4, perhaps, other transporters, bypassed the defect of tbnst4-null mutants. This may be sufficient for cell survival and for having the ability to synthesize glycoconjugates and/or other molecules to fulfill the role of TbNST4 in its absence.

Previous studies have shown that enzymes involved in nucleotide sugar biosynthesis in T. brucei such as UDP-Gal (59, 61, 78), GDP-Fuc (62), GlcNAc (63), and GDP-Man are essential for cell growth, but none of them were shown to cause pathogenicity changes in BSF T. brucei. We speculate that the function of the totality of TbNSTs is essential for cell growth and that inhibition of a specific or multiple tbnst involved in glycosylation of essential proteins for virulence will change the parasites’ infectivity. We will next focus on the KO of other identified TbNSTs, individually and combinatorially to determine their biological roles in this parasite.

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