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Characterization of an African trypanosome mutant refractory to lectin-induced death

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ABSTRACT

Incubation of African trypanosomes with the lectin concanavalin A (conA) leads to alteration in cellular DNA content, DNA degradation, and surface membrane blebbing. Here, we report the generation and characterization of a conA-refractory *Trypanosoma brucei* line. These insect stage parasites were resistant to conA killing, with a median lethal dose at least 50-fold greater than the parental line. Fluorescence-based experiments revealed that the resistant cells bound less lectin when compared to the parental line. Western blotting and mass spectrometry confirmed that the resistant line lacked an N-glycan required for conA binding on the cellular receptors, EP procyclin proteins. The failure to N-glycosylate the EP procyclins was not the consequence of altered N-glycan precursor biosynthesis, as another glycosylated protein (Fla1p) was normally modified. These findings support the likelihood that resistance to conA was a consequence of failure to bind the lectin trigger.

1. Introduction

African trypanosomes are the causative agent of sleeping sickness in man and nagana in cattle. These protozoan parasites have major life stages within distinct hosts, infecting both mammals and the tsetse fly insect vector. The interaction of parasites with one another and the host is critical for successful completion of the complex lifecycle, as development is carefully regulated in an environment-specific fashion.

Growing evidence suggests that the two major life stages, the procyclic insect form (PF[1]) and the mammalian bloodstream form (BSF), can respond to external and internal stimuli to trigger a cascade of cellular events leading to cell death that are superficially similar to metazoan apoptosis [1,2]. External stimuli include the lectin conA in PF cells [3,4] and prostaglandin D in BSF parasites [5]. Internal stimuli include endoplasmic reticulum (ER) stresses that alter protein expression [6].

These stresses yield cellular responses that are similar to that observed in higher eukaryotes that are unable to resolve unfolded protein-based stress through the unfolded protein response (UPR) pathway. In those cells, continued UPR stress triggers programmed cell death (PCD) via the activation of caspases [7,8]. The noted absence of clear homologs to the caspases involved suggests that the phenotypes that are similar to the PCD-based metazoan program are due to "incidental death" [9].

The lectin conA binds to glycosylated surface molecules on the PF parasite and can be lethal to the trypanosome [3,10,11]. The conA receptors, members of a class of surface molecules called EP procyclins, have a homogenous N-glycan found near the N-terminus that conA binds. Parasites that lack glycosylated EP procyclins due to genetic knockout or knockdown are resistant to conA killing [11,12], as are mutant parasites that produce defective N-glycans, indicating that conA binds to the N-glycan on EP procyclins [10]. ConA does not recognize a second class of procyclins that are expressed in a developmentally regulated fashion. This class of molecules, GPEET procyclins, lacks N-glycosylation, explaining the failure to bind conA.

Here, we report the isolation of a conA-resistant PF parasite cell line by chronic exposure to the lectin. Biochemical assessment of the line, clone 3B2, revealed that these cells are highly resistant to

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[1] The abbreviations used are: BSF, bloodstream form; conA, concanavalin A; GPI, glycosylphosphatidylinositol; IF, immunofluorescence; PCD, programmed cell death; PF, procyclic form; OST, oligosaccharyltransferase; SLS, spliced leader RNA silencing; UPR, unfolded protein response; vPBS, Voorheis's modified PBS; VSG, variant surface glycoprotein.

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conA killing, expressing EP procyclins that lack the characteristic N-glycan required for lectin binding. Notably, another N-glycosylated protein appears normally modified. In an effort to identify the genetic basis for the mutants, the protein coding regions of three candidate oligosyltransferase genes (*STT3A*, *STT3B*, and *STT3C*) that have been implicated in N-glycosylation of *Trypanosoma brucei* surface proteins were sequenced and found to lack mutations. Further, there were no marked changes in expression of the three genes in the 3B2 line, suggesting mutations in other loci are responsible for the phenotype.

2. Materials and methods

2.1. Trypanosome growth and selection for ConA resistance

PF 29-13 were grown in SDM-79 as described [13,14]. To select for parasites that were spontaneously resistant to conA binding, lyophilized conA (Sigma, St. Louis, MO) was hydrated in cytomix [15] supplemented with 1 mM $MnCl_2$ (cytoM) and incubated at a final concentration of 2 μg/mL overnight with PF parasites. Agglutinated parasites were removed by slow speed centrifugation (40*g*, 10 min) and survivors allowed to recover 24 h. This process was repeated three times, and then the concentration of conA increased to 5 μg/mL and then 10 μg/mL. Survivors were cloned by dilution and characterized.

2.2. Flow cytometry and cell staining with fluorescein-conjugated conA

Cells were labeled with fluorescein-conjugated conA (conA-FITC, Sigma, St. Louis MO) as described [12]. Cells (1×10^6) were resuspended in 1 ml cytoM containing 10 μg/ml conA-FITC (Sigma, St. Louis, MO). After 15 min at RT, trypanosomes were applied to a FACScan flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, NJ) and 10,000 cells analyzed per sample.

Lectin staining of parasites for microscopy was slightly modified from the previously described method [10]. Briefly, cells were adhered to poly L-lysine coated slides and fixed and permeabilized with 4% paraformaldehyde/0.5% formaldehyde (20 min, 4 °C). Parasites were then incubated with conA-FITC at 0.5 μg/mL (30 min, 4 °C) in cytoM and washed. Cells were visualized with a Zeiss Axioskop microscope.

2.3. MALDI-TOF-MS analysis of procyclins

For mass-spectrometry analysis, procyclins were purified from freeze-dried parasites (1×10^8) by sequential extraction with organic solvents [16]. To remove GPI anchors from polypeptides, dry butanolic extracts were dephosphorylated, dried, and resuspended in TFA (0.1%). An aliquot ($\sim$0.5 μL; $\sim 5 \times 10^6$ parasite equivalents) was co-crystallized with sinapinic acid in 70% acetonitrile, 0.1% TFA and analyzed by MALDI-TOF-MS. Data collection was in linear mode on a PerSeptive Biosystems Voyager-DE mass spectrometer (located at the Applied Biosystems Mass Spectrometry Facility of the Johns Hopkins University School of Medicine). The accelerating voltage was 2500 V, grid voltage was set at 91%, with an extraction time delay of 100 ns. Data were collected manually at 200 shots per spectrum, with laser intensity set at 2800. To confirm assignments, GPI anchors were removed by HF, and HF-treated samples ($\sim 5 \times 10^7$ parasite equivalents) were submitted to mild acid hydrolysis with 40 mM TFA for 20 min at 100 °C, which has been shown to break Glu-Pro bonds of EP procyclins [16]. Following treatment, MALDI-TOF-MS (as described above [16]) was used to confirm assignment (data not shown).

2.4. Analysis of N-glycan biosynthetic precursors

Lipid-linked oligosaccharides and other glycolipids were labeled in a cell-free system as described [17,18]. Briefly, washed cell lysates were supplemented with 5 mM $MnCl_2$ and 1 mM DTT, incubated with GDP[3,4-^{3}H]Man (0.5 μCi/mL, 18.3 Ci/mmol; PerkinElmer Life Sciences) and 2 mM UDP-GlcNAc for 5 min. Tunicamycin (0.8 μg/mL, Boehringer Mannheim, Indianapolis IN) was added to facilitate the identification of the dolichol-linked N-glycan precursor. The reaction was continued after the addition of 1 mM GDP-Man (20 min at 27 °C) and then terminated by addition of $CHCl_3/CH_3OH$ (1:1, v/v) to yield $CHCl_3/CH_3OH/H_2O$ in a 10:10:3 (v/v/v) ratio. Insoluble material was removed by centrifugation and organic supernatants dried under nitrogen. Lipids were extracted by addition of equal volumes of n-butanol and water, with the organic upper phase removed to a new tube. The lower aqueous phase was re-extracted twice with water-saturated n-butanol and the organic phases pooled and dried. Samples were then analyzed on silica gel 60 TLC plates (Merck) with plates developed in $CHCl_3/CH_3OH/H_2O$ (10:10:3, v/v/v), dried, and sprayed with En^{3}Hance (DuPont) and exposed to film at −80 °C.

2.5. Western blotting

To analyze EP-procyclins, membrane proteins from 4×10^6 cell equivalents were prepared as described [10] and probed with monoclonal antibody TBRP1/247 (Cedarlane Laboratories, Ontario Canada). Fla1p was detected from 2×10^7 cell equivalents that were either mock treated or incubated with 1U Endo H (24 h, 37 °C, 500 U/mL, New England Biolabs, Beverly MA). Proteins were then probed with anti-Fla1p antibody (1:300 dilution, a gift of Dr. G.A.M Cross, Rockefeller University).

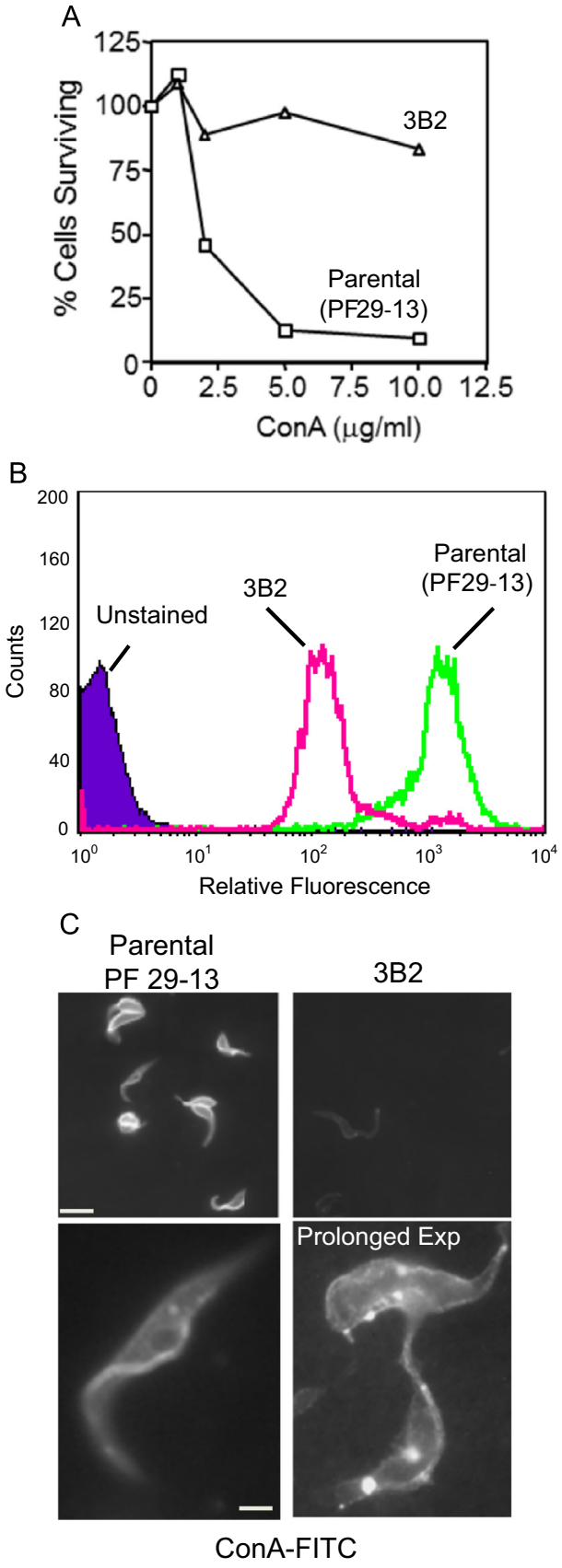
2.6. Genetic analysis of the STT3 alleles

The protein coding regions of the three *STT3* genes (*STT3A*, *STT3B*, and *STT3C*) were amplified and sequenced from both parental and 3B2 lines using primers complementary to unique UTRs of the genes. Sequences were then compared to one another (to resolve strain-specific polymorphisms) as well as to the annotated genome (TriTrypDB, http://tritrypdb.org/tritrypdb/). For RT-qPCR, total RNA from parental and 3B2 cells was extracted using the Bio-Rad Laboratories Aurum Total RNA Mini Kit according to the manufacturer's directions. Reverse transcription and quantitative PCR was performed using the Bio-Rad iTaq Universal SYBR Green One-Step Kit employing primers designed to the variable regions of the *STT3* genes [19]. Relative expression was scored using the comparative CT($2^{-\Delta\Delta CT}$) method based on expression of the *PI3K-like* reference gene, as described [19,20]. Statistical analysis to test for difference in expression was performed by *t*-test.

3. Results

Isolation of a trypanosome line that is highly resistant to conA killing. The lectin conA triggers a cell death response in PF trypanosomes [3,11]. As has been previously described, we found that incubation of parasites with the protein causes a rapid clumping, loss of morphology, and disruption of nucleic acid organization (data not shown). To further explore the conA killing pathway in trypanosomes, we pursued the isolation of conA resistant parasites. To accomplish this, parasites were cultured with increasing concentrations (1, 2, 5, and 10 μg/mL) of conA, allowing the culture to recover for 24 h between increase in treatments. Please note, the EC_{50} value (the concentration required to reduce cell survival 50% after 48 h) was about $\sim$1–2 μg/mL conA for the parental line.

Survivors were cloned and we initially characterized two lines. Since the clones had behavior and biochemical characteristics that were nearly indistinguishable, we have focused on a single clone, 3B2. This clone was remarkably resistant to conA killing, essentially being refractory to the toxic lectin at all concentrations tested. This is in comparison to the EC_{50} of the parental line (Fig. 1A). Further, 3B2 cells continued to grow and have normal morphology in the continued presence of the highest conA concentration tested (50 μg/mL). Culturing for three months in the absence of selection with conA did not lead to reversion to a parental phenotype.



Analysis of live trypanosomes by flow cytometry confirmed that clone 3B2 had a markedly reduced (though not completely ablated) affinity for conA when compared to parental 29-13 cells (Fig. 1B). ConA-FITC labeled the entire parasite cell surface, particularly the flagellum in the parental parasites (Fig. 1C, top left). Labeling of the 3B2 cells was dramatically reduced, with conA-FITC signal difficult to detect in an exposure equal to that used to visualize the parental cell staining. Using an increased image acquisition time, faint punctate staining was observed (Fig. 1C, bottom right), similar to that found in other conA-resistant mutant parasites [10] and detectable to a much lesser extent in some parental cells. Additionally, weak membrane labeling was observed.

How do 3B2 cells avoid the toxicity of conA? ConA kills trypanosomes following binding to glycosylated EP procyclins [11]. ConA-resistant parasites have been described, with lectin-resistant parasites avoiding conA toxicity through different mechanisms including expression of EP procyclins with altered N-glycan occupancy, disrupted N-glycans biosynthesis, EP procyclin gene deletion, or surface glycoprotein switching that results from RNAi of glycolytic genes [10–12,21,22]. To explore the remarkable conA resistance found in clone 3B2 cells, we first assessed the expression of EP procyclin by western blot. The parental PF 29-13 parasites expressed the characteristic heterogeneous "smear" of EP procyclins predominantly between ~40-44 kDa (which results from the heterogeneity of the glycosylation of the GPI anchor) (Fig. 2A). Likewise, EP procyclins from clone 3B2 have a heterogeneous pattern, but the most intense cross-reactivity had a faster electrophoretic mobility (between ~38–42 kDa) than that found in the parental sample.

The differences in mobility between the parental and clone 3B2 EP procyclins suggested that conA resistance could result from some difference in the EP-procyclin molecule. To assess this, we analyzed the parasite's procyclin repertoire by MALDI-TOF mass spectrometry (Fig. 2B). This method identifies both the procyclins that are expressed in a population as well as their glycosylation state [16]. The parental PF 29-13 cells expressed a variety of procyclins, including EP1-1, EP1-2, and EP3, with all decorated by a homogeneous high mannose-type N-glycan near the N-terminus. GPEET procyclin was not detected in the parental line. Clone 3B2 likewise did not express detectable GPEET procyclin. Interestingly, the EP procyclins from 3B2 cells that were detected lacked 1217.1 daltons of mass characteristic of the homogenous $Man_5GlcNAc_2$*N*-glycan found on EP1 and EP3. Because the method used to isolate procyclins for MALDI-TOF mass spectrometry

Fig. 1. PF clone 3B2 is highly resistant to conA killing. (A) Parental PF 29-13 and PF clone 3B2 parasites were incubated for 48 h in the presence of increasing concentrations of conA and cell densities determined with cell numbers plotted as a percentage of control untreated cells. The data presented here are representative of 4 independent experiments. (B) Live trypanosomes were incubated with 10 μg/ml fluorescein-conjugated conA for 15 min at RT in cytoM and then analyzed by flow cytometry (10,000 cells/assay). Parental PF 29-13 and clone 3B2 were analyzed, with the laser intensity adjusted to yield autofluorescence from unstained cells of ~5 relative fluorescence intensity units. (C) Microscopic analysis of conA binding. Fixed cells were incubate with conA-FITC (0.5 μg/ml) and images acquired with equal exposure times (top). For analysis of internal staining, parental cells (left, standard image acquisition time) or 3B2 cells (right, prolonged image acquisition time) were compared. Scale bar = 5 μm (upper) or 1 μm (lower).

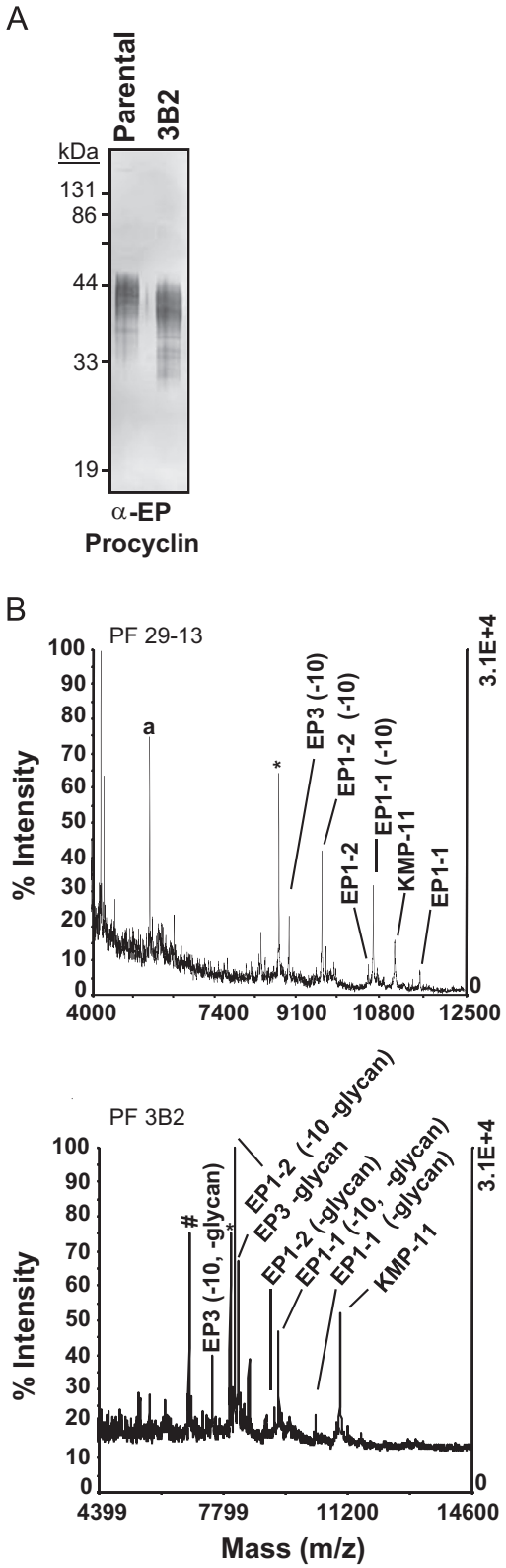


Fig. 2. EP procyclins from PF clone 3B2 lack N-terminal glycan modification. (A) Western blot analysis of proteins probed with monoclonal antibody TBRP1/247 to detect EP procyclins. (B) MALDI-TOF MS analysis of parental PF 29-13 (top) and PF 3B2 (bottom) procyclins. $[M-H]^-$ ions were identified using the assignments in [16]. An internal standard (insulin, 5733 Daltons, indicated by 'a') was added to the PF 29-13 sample. EP1-1, EP1-2, and EP3 have *m/z* of 11,531, 10,430, and 9723. Species indicated as (-10) lack ten amino acids from the N-terminus, a cleavage occurring during HF treatment [16]. The ion at *m/z* 8504 (indicated with *) is likely a contaminant (see [12]). The kinetoplast membrane protein-11 (KMP-11) has been found to contaminate procyclin (butanol) preparations. In the PF 3B2 spectrum, all of the detected procyclins lacked 1217.1 daltons characteristic of the homogenous N-glycan found on EP1 and EP3. The ion at *m/z* 6870 (indicated with #) is unknown.

partially purifies only GPI-anchored procyclins (which are removed prior to MALDI-TOF by hydrofluoric acid treatment), and the mass of all procyclin polypeptides (either full length proteins or mild acid-cleaved C-termini) was consistent with the presence of a C-terminal EtN-PO_4 group, it is likely that 3B2 cell procyclins are properly GPI anchored.

The 3B2 cell line may have a defect in N-glycan biosynthesis. Analysis of N-glycan and GPI precursor biosynthetic intermediates using a cell-free system revealed there were subtle differences in steady-state level of pre cursors between parental and 3B2 cells (Fig. 3A) [17], particularly in the unknown species migrating above the PP3 standard. Treatment with tunicamycin, which blocks N-glycan production by inhibiting Dol-PP-GlcNAc biosynthesis, supported the finding of minor differences between the parental and mutant samples. However, the impact on N-glycans may not be global. While the EP procyclins from the 3B2 line lacked detectable N-glycans, another less-abundant glycoprotein was not impacted. The flagellum-adhesion glycoprotein 1 (Fla1p) protein

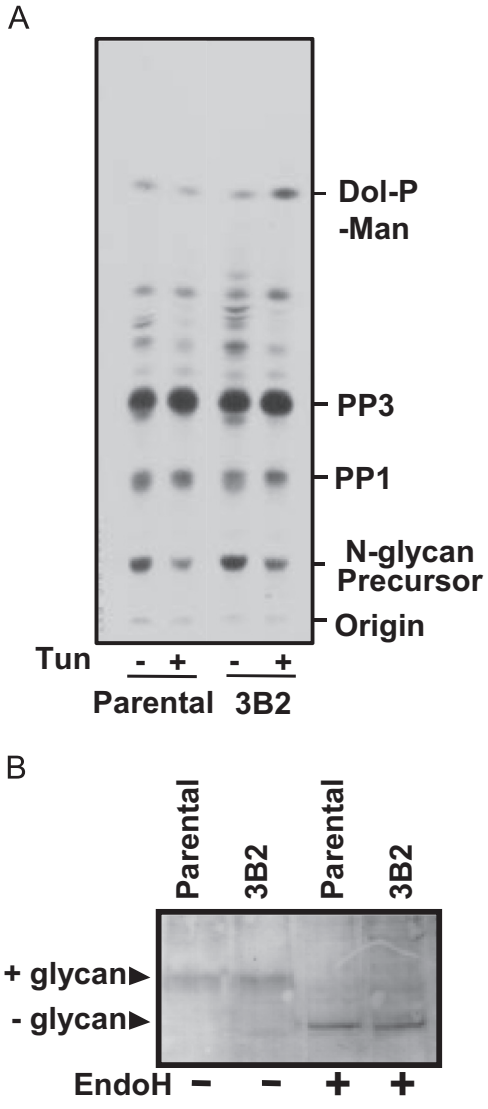


Fig. 3. (A) N-glycan and GPI biosynthetic precursors analysis of parental and 3B2 cells treated with or without tunicamycin (Tun), a Dol-PP-GlcNAc biosynthesis inhibitor. GDP-[^{3}H]Man-labeled products from a cell-free biosynthesis system were fractionated by TLC, and detected by autoradiography. Dol-P-Man, dolichol phosphoryl Man; PP1, ethanolamine phosphate Man_3GlcN-acylinositol *lyso*-phosphatidylinositol; and PP3, ethanolamine phosphate Man_3GlcN-acylinositol alkyl-acyl-phosphatidylinositol, GPI precursors; O, origin. (B) Fla1p western blot. Membrane proteins from parental and clone 3B2 were either mock treated or incubated with 1U Endo H and then probed with anti-Fla1p serum.

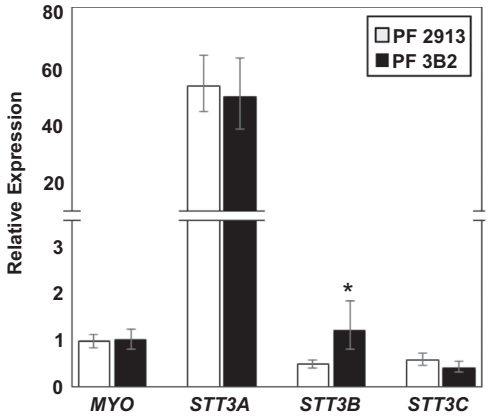


Fig. 4. RT-qPCR expression analysis of *STT3* isoforms in parental and 3B2 cells. Total RNA of parental and 3B2 cells lines (from 1×10^7 parasites) was reverse transcribed and then relative abundance of the cDNA from the myosin 1B gene (*MYO*, a control gene) and *STT3* alleles (*STT3A*, *STT3B*, and *STT3C*) scored using primers described in [19]. Relative expression for all genes was calculated with respect to the expression of the *PI3K-like* gene (Tb927.2.2260), which has been used previously in RT-qPCR analysis of *STT3* transcripts [19]. Values were determined from triplicate analysis of independent reactions. *, p value < 0.05.

[23] is a N-glycosylated transmembrane protein harboring five asparagine glycosylation sites [23]. In both the parental PF 29-13 and 3B2s, Fla1p was glycosylated, with a mobility of ~80 kDa that was reduced following treatment with endoglycosidase H (Endo H), an enzyme that removes only N-glycans (either high mannose or hybrid types) (Fig. 3B).

The failure of the 3B2 parasites to bind conA is a stable and heritable phenotype, suggesting the cells may have a mutation in a protein involved in glycan transfer to EP procyclins. Selection using lectins with high affinity for BSF glycans has been used to generate parasites lacking the triantennary oligomannose moiety on the BSF dominant surface molecule, VSG. These BSF parasites have a defect in the expression of the *TbSTT3B* gene, one of three related oligosaccharyltransferase (OST) genes (*TbSTT3A-C*) in the genome that may participate in asparagine-linked glycosylation [19,24]. To determine if the 3B2 line had a similar defect, the OST open reading frames encoded by the *TbSTT3* genes were cloned and sequenced from the genomes of both parental and mutant lines and compared to one another and to reference *T. brucei* genomes (http://tritrypdb.org/tritrypdb/). This effort demonstrated no differences between the parental and 3B2 lines in the STT3 protein coding regions. Analysis of the OST genes by RT-qPCR to assess changes in expression revealed that while the *STT3B* mRNA abundance was significantly increased (p-value < 0.05), there was no obvious lack of STT3 expression in the 3B2 line to explain the N-glycosylation defect (Fig. 4).

4. Discussion

T. brucei is covered by an important surface coat as it develops within its two hosts. In the mammalian bloodstream, 10^7 molecules of the GPI anchored variant surface glycoprotein (VSG) form a protective layer on the parasite's surface that thwarts host immune responses and provides the basis for antigenic variation. Within the insect vector, the parasite coat primarily consists of members of the procyclin family, including EP and GPEET procyclins. While there are fewer procyclin molecules per parasites (5×10^6) than VSG, the heterogeneously glycosylated GPI sidechain of procyclins likely forms a protective barrier for the surface of the parasites against both digestive enzymes and insect immune response.

Procyclins may additionally play a role in parasite adhesion to host tissues with the expression of different members of the procyclin family coinciding with the tropism in the fly. Indeed, when high concentrations of sugars were introduced simultaneously with parasites during a blood meal, insect infection was found to be reduced [25]. Further, the genetic ablation of the EP genes yields parasites that are morphologically indistinguishable for the parental cells but have a compromised ability to establish a heavy infection in the insect midgut [18]. These data suggest an interaction between parasite surface glycoproteins and insect lectins. The observation that ConA triggers a cell death response in the parasite may reflect an overstimulation of a signaling pathway that is normally induced by binding of authentic insect lectins.

The consequences of conA binding on parental cells include alteration in cellular DNA content due to disruption of replication, DNA degradation, surface membrane blebbing – all in the absence of loss of membrane integrity [26]. The 3B2 line lacks N-glycan modification of EP procyclins but has no obvious alterations in the OST genes implicated in transfer of glycan to surface molecules in the parasite. This is different than the recently described *T. brucei* BSF mutants resistant to carbohydrate binding agents in which the *STT3* genes expression was down-regulated or the *STT3* genes were recombined to produce enzymatically inactive proteins [19]. As an alternative explanation, this phenotype may reflect an authentic developmentally regulated change in the glycan decorations found on EPs during a heretofore-unrecognized PF lifecycle stage. Since the alteration is stable and heritable, the 3B2 clone could be irreversibly trapped in this stage due to a mutation in a gene that regulates development. Other explanations are possible including the presence of a mutation in a pathway distinct from the OST proteins that participates in glycan transfer to EP procyclins. Indeed, the punctate staining seen in the prolonged exposures of 3B2 cells may reflect an accumulation of a glycan intermediate.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.08.013.

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