Expression of a variant surface glycoprotein of *Trypanosoma gambiense* in procyclic forms of *Trypanosoma brucei* shows that the cell type dictates the nature of the glycosylphosphatidylinositol membrane anchor attached to the glycoprotein

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Procyclic forms of *Trypanosoma brucei* have been genetically modified to express the major metacyclic variant surface glycoprotein (VSG variant AnTat 11.17) of *Trypanosoma gambiense*. The VSG is expressed in an intact membrane-bound form that can be detected over the entire plasma membrane, together with procyclin, and as a series of lower-molecular-mass fragments that are mostly soluble degradation products. The presence of degraded VSG in the cells and the culture medium suggests that VSG is not efficiently processed and/or efficiently folded when expressed in procyclic cells. The level of procyclin expressed on the surface of these cells is slightly reduced, although there is no difference in procyclin mRNA levels. The intact membrane-bound form of the VSG is N-glycosylated with oligomannose structures and contains a glycosylphosphatidylinositol (GPI) membrane anchor that can be biosynthetically labelled with [³H]ethanolamine. The anchor is sensitive to mammalian GPI-

specific phospholipase D but, like the anchor of procyclin, it is resistant to the action of bacterial phosphatidylinositol-specific phospholipase C. This pattern of phospholipase sensitivity suggests that the GPI anchor acquired by VSG when expressed in procyclics is acylated on the inositol ring and therefore resembles a procyclic procyclin-type anchor rather than a trypomastigote VSG-type anchor with respect to the lipid structure. The VSG expressed in procyclics was sensitive to the action of a mixture of sialidase, β -galactosidase and β -hexosaminidase, suggesting that the VSG GPI anchor also contains a sialylated poly-lactosamine side-chain modification similar to that described for procyclin. These results indicate that the nature of the protein expressed has little influence on the post-translational modifications performed in the secretory pathway of procyclic trypanosomes.

INTRODUCTION

In the different subspecies of the *Trypanosoma brucei* group, the variant surface glycoprotein (VSG) is the most abundant surface protein of the metacyclic and bloodstream trypomastigote forms whereas the VSG coat is replaced by procyclin in the procyclic forms. These antigens are never expressed simultaneously, except briefly during the transition between forms. Whereas it is known that the VSG coat allows the parasite to escape the antibody response of the host through continuous variation of its exposed epitopes, the role of procyclin is still unclear. Both proteins form homogeneous surface coats and are attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, but they differ completely in sequence and structure. Procyclin adopts an extended rod-like configuration as it contains a long domain consisting of Glu-Pro repeats [1,2], whereas VSG exists as a dimer in which each monomer is made of two distinct domains, a variable N-terminal region folded into two α -helical bundles with a loop exposing the surface epitopes and a more conserved, and disulphide-constrained, C-terminal domain close to the plasma membrane [3,4]. In addition, the GPI anchor structures

of these glycoproteins are substantially different. The VSG anchor contains a dimyristoylphosphatidylinositol moiety and an α Gal side chain [5] whereas procyclin contains predominantly a 1-*O*-stearoyl-2-lysophosphatidylinositol moiety [6,7] and a large and heterogeneous sialylated poly-lactosamine-containing side chain [8]. The differences in the lipid structures are reflected in the GPI anchor precursors of the trypomastigote [9] and procyclic forms [10,11] and may be due to the down-regulation of the fatty acid-remodelling enzymes [12] and/or the inositol deacylase in procyclic forms [13]. The differences in the carbohydrate side chains may be due to stage-specific expression of the relevant glycosyltransferases and/or the influence of the glycoprotein structure on the processing of GPI anchor in the endoplasmic reticulum and/or Golgi apparatus. Interestingly, the expression of VSG in mammalian COS cells has revealed that the C-terminal signal peptide that directs the addition of the GPI anchor in trypomastigotes does not function in mammalian cells [14]. However, this issue has not been previously addressed in the different developmental stages of trypanosomes.

The metacyclic VSGs cover the few trypanosomes that invade the mammalian blood during the blood meal of the tsetse fly, and

Abbreviations used: DTT, dithiothreitol; FITC, fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; PI, phosphatidylinositol; PLC, phospholipase C; PLD, phospholipase D; VSG, variant surface glycoprotein; UTR, untranslated region.

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therefore represent the best theoretical candidates for vaccination against the parasite. This suggestion is strengthened by the observation that the repertoires of metacyclic VSGs are relatively limited, although they appear to evolve in *T. brucei* and *T. rhodesiense* [15]. Remarkably, the VSG repertoires of *T. gambiense* are both less extensive and much more conserved than those of other subspecies of *T. brucei* [16–19]. Despite our limited knowledge of the metacyclic VSG repertoires of *T. gambiense*, one may speculate that they are sufficiently limited and conserved to represent possible vaccine candidates. The major metacyclic VSG of the AnTaR 11 serodeme of *T. gambiense* (AnTat 11.17) has been characterized [20]. The AnTat 11.17 gene is unique and it is present in all typical *T. gambiense* strains from West Africa (see [21] for a discussion on the status of the *T. gambiense* subspecies), but absent from most *T. brucei* or *T. rhodesiense* stocks examined ([20]; C. D. Do Thi, P. Paindavoine and E. Pays, unpublished work).

The aim of this work was to produce *T. brucei* procyclic forms expressing the AnTat 11.17 VSG through targeted homologous recombination [22–24] and to analyse the location and post-translational processing of this trypomastigote-specific glycoprotein when expressed in procyclic trypanosomes.

MATERIALS AND METHODS

Construction of pTSA-11.17PRO

The 1.6 kb *EcoRI* fragment containing the complete AnTat 11.17 VSG cDNA [20] was subcloned in M13 in order to introduce, by site-directed mutagenesis, a *XhoI* and a *XbaI* site just before and just after respectively the open reading frame. This *XhoI*–*XbaI* fragment was then ligated into the pTSA-HYG2 vector [25], which was previously modified by insertion between the *AccI* and *EcoRV* sites of the complete 3'-untranslated region (UTR) of the α -procyclin gene of the PARP A locus of *T. brucei*. This 3'-UTR fragment was obtained by PCR amplification of a 555 bp region between the stop codon and a downstream *ApaI* site, followed by subcloning in the pSL1180 vector (Pharmacia) and excision by *AccI* and *SmaI*. The resulting plasmid, pTSA-11.17PRO, is shown in Figure 1.

Genomic targeting of the plasmid constructs

The procyclic trypanosomes (EATRO 1125 strain) were grown in SDM-79 medium [26] supplemented with 15% heat-inactivated fetal calf serum. They were harvested at mid-exponential phase and washed once in Zimmerman post-fusion medium [27]. Then 10^7 cells were resuspended in 500 μ l of the same solution and mixed with 30 μ g of plasmid linearized by *MluI* cleavage. Electroporation was performed by two pulses of 1.5 kV at 25 μ F using a Bio-Rad gene pulser. Electroporated trypanosomes were kept on ice for 5 min and inoculated into 4 ml of culture medium. At 48 h after electroporation, hygromycin was added to the culture medium, first at 25 μ g/ml and then at 100 μ g/ml 5 days later. Transgenic trypanosomes could be detected 2–3 weeks after electroporation.

Antibodies

AnTat 11.17 infection serum

The serum was prepared from rabbits 6 days after the intravenous injection of 10^6 slender forms from the AnTat 11.17 clone of *T. gambiense*.

AnTat 11.17 hyperimmune serum

The VSG was released from AnTat 11.17 trypanosomes by incubation of 2×10^9 cells for 16 h at 4 °C with gentle shaking in 10 ml of buffer containing 0.3% glucose, 1 mM *p*-chloromercuriphenylsulphonic acid, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin and 123 mM sodium phosphate, pH 5.5 [28]. The supernatant was recovered after two successive centrifugations at 4 °C, first for 10 min at 3000 *g* then for 1 h at 165 000 *g*. After freeze-drying, the VSG was dissolved in 1 ml of PBS, submitted to preparative, 10% PAGE and eluted from the gels. A New Zealand White rabbit was immunized three times at 2-week intervals with this purified antigen (successively 150, 100 and 100 μ g). This serum was diluted 50 000-fold for incubation with Western blots.

Anti-procyclic antibodies

Monoclonal anti-procyclic antibodies against procyclic with a Glu-Pro repeat domain (EP-procyclic) were purchased from Cedar Lane Laboratory and diluted 1000-fold for use on Western blots. Polyclonal rabbit antibodies against procyclic with a Gly-Pro-Glu-Glu-Thr repeat domain (GPEET-procyclic), a gift from Dr. Isabel Roditi, University of Berne, Berne, Switzerland, were diluted 1000-fold for use on immunoblots.

Protein extraction

Trypanosomes (10^6 cells/ μ l) were lysed in buffer containing 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 50 μ M Pefabloc SC (Boehringer), 10 μ M E64 (Boehringer), 1 μ M pepstatin and 50 mM Tris/HCl, pH 7.4. Samples of the whole-cell lysates were mixed with loading buffer, boiled for 3 min and analysed by SDS/PAGE followed by Coomassie Blue staining or Western blotting.

Partitioning of membrane proteins by phase separation in Triton X-114

Trypanosomes (10^8) were homogenized at 0 °C in 550 μ l of buffer containing 150 mM NaCl, 0.05% Triton X-100, 50 μ M Pefabloc SC, 10 μ M E64, 1 μ M pepstatin and 50 mM Tris/HCl, pH 7.5. After incubation at 0 °C with 50 μ l of precondensed 12% Triton X-114, the extracts were centrifuged for 10 min at 10 000 *g* and submitted to phase separation [29]. The 50 μ l detergent phases were diluted to 1.6 ml with the homogenization medium and divided into two samples for incubation with and without *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC). Incubations were performed with 0.5 unit of phospholipase/ 10^7 trypanosomes, for 30 min at 37 °C in the presence of 0.05% Triton X-100. After incubation, one-eleventh volume of 12% Triton X-114 was added to each sample, and the phase separation was repeated. The aqueous and detergent phases were finally precipitated in acetone in the presence of 100 μ g of RNase as carrier.

Immunofluorescence

The trypanosomes were collected by centrifugation and fixed overnight at 4 °C in PBS containing 2% (v/v) formaldehyde and 0.05% (v/v) glutaraldehyde. After washing in PBS containing 2% BSA, the cells were applied to microscope slides and dried for 1 h at 37 °C. The slides were sequentially incubated at 20 °C with rabbit anti-(AnTat 11.17 VSG) antibodies for 60 min (either infection serum or hyperimmune serum diluted 25- and 50-fold in PBS respectively) with PBS for 15 min, with 50-fold diluted fluorescein isothiocyanate (FITC)-labelled donkey anti-rabbit

Figure 1 Genomic targeting and transcription of pTSA-C (C) and pTSA-11.17PRO (V)

The probes indicated below the blots by 11.17 and HYG^R are a 1.2 kb *SacI*–*HpaI* fragment from the AnTat 11.17 cDNA [20] and a 723 bp *EcoRI*–*ScaI* fragment from the HYG^R gene [25] respectively. The extent of each probe is indicated under the map by the bars marked 11.17 and H respectively. (A) Southern blots of genomic DNA (1 $\mu\text{g}/\text{lane}$) of the transformed procyclic forms, after digestion by *Bam*HI (Ba) or *Bam*HI + *Mlu*I (M1). The fragments labelled with an arrow and a dot are discussed in the text. (B) Northern blots of 1, 3 and 9 μg (lanes 1, 2 and 3 respectively) of polyadenylated RNA from the transformed procyclic forms. The procyclin (PROC) and actin (ACTIN) gene probes indicated below the blots are a 1.3 kb *ScaI*–*EcoRI* fragment from the pAP2 plasmid [26] and a 1.5 kb *SaI*/I fragment from the pBR-actin plasmid [34] respectively. In the three blots, the top bands represent mRNA-processing intermediates. The map shows the pTSA-11.17PRO construct in its genomic context, between tandem repeats of the α - and β -tubulin genes. The *Mlu*I site used for linearization is indicated by an encircled M. The AnTat 11.17 gene is located downstream of the procyclin promoter and splice acceptor site (indicated by an encircled 1), and upstream of the procyclin 3'-UTR and polyadenylation site (indicated by an encircled 2). The procyclin splice acceptor site is also present upstream of the HYG^R gene (indicated by an encircled 3).

secondary antibody (Amersham Braunschweig) for 45 min and finally with PBS for 15 min. The slides were observed under a Leitz fluorescence microscope and photomicrographs were taken with phase-contrast and fluorescent illumination at $\times 12000$ magnification.

Electron microscopy and gold immunolabelling

The trypanosomes were incubated at 0 °C in PBS containing 1 % BSA for 15 min and then in a mixture of rabbit anti-(AnTat 11.17 VSG) antibodies and monoclonal anti-EP-procyclic antibodies, both diluted 20-fold in PBS containing 1 % BSA, for 45 min. After two washes with PBS containing 1 % BSA, the cells were fixed for 15 min at room temperature in a mixture of 4 % formaldehyde (prepared from paraformaldehyde) and 0.1 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The cells were rinsed twice with PBS and washed for 15 min with PBS containing 0.1 M glycine before being incubated for 90 min at

room temperature with a mixture of goat anti-rabbit IgG (H + L) conjugated to 5 nM gold particles (GAR G5; Amersham) and goat anti-mouse IgG (Fc) conjugated to 10 nm gold particles (GAM IgG G10), both diluted 50-fold in PBS containing 1 % BSA. After three washes at room temperature with PBS-containing 1 % BSA and 0.05 % Tween 20, the cells were fixed again in 2.5 % glutaraldehyde diluted in cacodylate buffer, washed and post-fixed in 2 % OsO_4 in the same buffer. The cells, clustered in agar, were dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained successively with uranyl acetate and lead citrate, and observed under an AEI 6B electron microscope at 60 kV.

[^3H]Ethanolamine labelling of trypanosomes and immunoprecipitation

Procyclic cells in the exponential phase of growth were washed twice in SDM-79 medium containing 10 % dialysed fetal calf

serum and resuspended in the same medium containing 50 mCi/ml [^3H]ethanolamine (17.9 Ci/mmol; DuPont–NEN) at 3.5×10^7 cells/ml. The cells were labelled under aseptic conditions for 32 h at 27 °C in a shaking water bath, counted, harvested by centrifugation and washed three times with ice-cold PBS. A portion of the cells was lysed at 5×10^8 cells/ml in SDS sample buffer containing 0.1 M dithiothreitol (DTT), boiled and sonicated for 10 min to produce a total cell lysate. The majority of the cells (4×10^8 cells) were lysed in 200 mM Tris/HCl, pH 7.5, containing 2% Nonidet P40, 20 mM EDTA, 0.1 mM PMSF, 0.1 mM Tos-Lys- CH_2Cl , and 1 mg/ml leupeptin (1 ml final volume) and incubated for 1 h on ice. The extract was clarified in a microcentrifuge (10 min in the cold) and the supernatant was split into two equal fractions (samples A and B). The samples were adjusted to the composition of immunoprecipitation (IP) buffer (50 mM Tris/HCl, pH 7.5, 0.5% Nonidet P40, 5 mM EDTA, 0.1 mM tosyl-lysylchloromethane ('TLCK'), 1 mg/ml leupeptin, 0.75 mg/ml BSA, 0.25 M NaCl). Sample A was mixed with 50 μl of a 50% suspension of anti-(AnTat 11.17 VSG) antibodies (about 10 μg of relevant IgG) coupled to CNBr-activated Sepharose 4B beads (Pharmacia) according to the manufacturers' instructions. Sample B was mixed with 50 μl of a 50% suspension of preimmune antibodies coupled to Sepharose, and, in a third sample (sample C), IP buffer without cell lysate was mixed with 50 μl of a 50% suspension of anti-(AnTat 11.17 VSG) antibodies coupled to Sepharose.

The samples were incubated for 1 h at 4 °C with continuous mixing and the beads were washed three times with 1 ml of 50 mM Tris/HCl, pH 7.5, containing 0.5% Nonidet P40, 0.5 M NaCl, 0.5 mg/ml BSA and 5 mM EDTA, four times with 1 ml of 50 mM Tris/HCl (pH 7.5)/0.5% Nonidet P40 and three times with 1 ml of water. After the last washing step, the beads were boiled three times with 25 μl of $2 \times$ SDS sample buffer without DTT. The combined supernatants were analysed by SDS/PAGE and Coomassie staining, by Western blotting with anti-(AnTat 11.17 VSG) antibody and by fluorography after impregnation of the gel with En 3 Hance (DuPont–NEN).

GPI-specific phospholipase D (GPI-PLD) treatment and product fractionation

pTSA-11.17PRO transformed procyclic cells (4×10^{10}) were pelleted, washed twice with Tris-buffered saline and lysed at 5×10^9 cells/ml in buffer containing 2% Triton X-114, 150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 1 mM PMSF, 2 μg /ml leupeptin and 0.2 mM Tos-Lys- CH_2Cl on ice for 2 h. The extract was clarified in a microcentrifuge (10 min in the cold) and the supernatant was transferred to another Eppendorf tube and subjected to Triton X-114 phase separation by the method of Bordier [29]. The detergent phase was washed twice with pre-equilibrated aqueous phase and diluted to below the critical micelle concentration of Triton X-114 using 5% propan-1-ol/0.1 M ammonium acetate. The sample was loaded at 2 ml/h on to an octyl-Sepharose column (1 cm $\times$ 20 cm) pre-equilibrated with 5% propan-1-ol/0.1 M ammonium acetate. The column was washed with the same buffer at 4 ml/h until all the Triton X-114 (monitored by UV absorbance at 275 nm) was removed. The column was subsequently eluted using a propan-1-ol gradient from 5 to 60% at 4 ml/h. The propan-1-ol concentration of the collected 1 ml fractions was determined by measuring the refractive index. The fractions were dried and redissolved in 30 μl of 5% propan-1-ol. Aliquots of each fraction were dot-blotted on to two nitrocellulose membranes, one of which was stained with anti-(AnTat, 11.176 VSG) antibody and the other one with anti-EP-procycloclon antibody. The blots were developed using

ECL Western-blotting detection reagents (Amersham), according to the manufacturer's instructions. Fractions positive for VSG were pooled, dried, redissolved in 100 μl of water and split into two equal fractions. Purified *T. brucei* GPEET-procycloclon (1 nmol), kindly provided by Achim Treumann (University of Dundee) was added to each fraction as an internal control. Samples of purified procycloclon alone were also prepared. All samples were dried and redissolved in 20 μl of 20 mM Tris/HCl, pH 7.4, containing 0.1 mM CaCl_2 and 0.008% Triton X-100 and incubated with and without 2 μl of human serum as a source of GPI-PLD. All samples were overlaid with 1 μl of toluene and incubated overnight at 37 °C. Aliquots (before and after digestion) were taken from each sample for SDS/PAGE and anti-VSG Western blotting to check for VSG degradation. The samples were diluted to 400 μl with water and filtered through a 0.2 μm membrane before injection on to a Vydac wide-pore C_4 reverse-phase HPLC column (15 cm $\times$ 0.46 cm). The column was eluted with an acetonitrile gradient from 0 to 60% at a flow rate of 1.5 ml/min. Fractions (1 ml) were collected and the elution positions of GPI-PLD-treated procycloclon and VSG were analysed on nitrocellulose membranes using specific antibodies, as described above.

Endoglycosidase digestions

Transformed procyclics were washed twice with PBS, lysed at 2×10^9 cells/ml in solution containing 1% SDS, 0.1 M DTT, 1 mM PMSF, 0.4 mM Tos-Lys- CH_2Cl and 2 μg /ml leupeptin, vortex-mixed and boiled for 5 min. Aliquots (2.5 μl) of this lysate were mixed with 10 μl of 2.5% Nonidet P40, 5 μl of digestion buffer (for endoglycosidase H treatment 400 mM ammonium acetate, pH 5.5, 1 mM PMSF, 0.4 mM Tos-Lys- CH_2Cl , 400 mM DTT; for N-glycosidase F treatment 200 mM Tris-acetate, pH 7.2, 40 mM EDTA, 200 mM DTT) and 2.5 μl (2.5 m-units) of recombinant endoglycosidase H (Boehringer-Mannheim) or 2 μl (1000 units) of PNGase F from *Flavobacterium meningosepticum* (New England Biolabs). After digestion at 37 °C overnight, the samples were spun in a microcentrifuge, and 20 μl of $2 \times$ SDS sample buffer was added to each supernatant. The samples were brought to a final concentration of 0.1 M DTT, boiled for 5 min and analysed by SDS/PAGE followed by Western blotting and staining with anti-(AnTat 11.17 VSG) antibody. As controls, 20 μg aliquots of VSG 221 (a type-2 VSG that contains two N-glycosylation sites) were subjected to the same enzyme treatments and analysed by SDS/PAGE followed by Coomassie Blue staining.

Exoglycosidase digestions

Transformed procyclics were washed twice with PBS and lysed at 2×10^9 cells/ml in 1% SDS containing 1 mM PMSF, 0.2 mM Tos-Lys- CH_2Cl and 2 μg /ml leupeptin. Aliquots (3 μl) of this lysate were added to a mixture of enzymes (final volume 50 μl) containing 0.2 unit of *Arthrobacter ureafaciens* sialidase (Oxford GlycoSystems), 0.05 m-unit of bovine testis β -galactosidase (Boehringer-Mannheim) and 1 unit of jack-bean β -N-acetylhexosaminidase (Oxford GlycoSystems) and incubated overnight at 37 °C. After digestion, the samples were boiled for 5 min and analysed by SDS–PAGE (10% gel) followed by Western blotting and staining with anti-(AnTat 11.17 VSG) antibody. As a control, 100 pmol of purified GPEET-procycloclon was subjected to the same enzyme treatment and analysed by SDS/PAGE 15% gel stained with 1-ethyl-2-[3-(1-ethylnaphthol-[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-*d*]thiazolium bromide (Stains-All; Sigma) as described in [30,31].

The map in Figure 1 shows the structure of the plasmid construct that led to the highest synthesis of VSG mRNA, and which was used in this work. This plasmid, pTSA-11.17PRO, contains successively the procyclin promoter, the AnTat 11.17 open reading frame flanked by the 5'- and 3'-UTRs of the α -procyclin gene from the PARP A locus, the gene for resistance to

Figure 3 Western-blot analysis of proteins from transfected procyclic cells after Triton X-114 phase separation

A whole-cell lysate was subjected to phase separation and analysed by SDS/PAGE (10% gel) followed by Western blotting and staining with anti-(AnTat 11.17 VSG) antibody. Lane 1, aqueous phase; lane 2, detergent phase.

hygromycin and a 640 bp fragment containing the β - α tubulin intergenic region.

Genomic targeting and transcription of the VSG gene

After linearization by digestion with *Mlu*I, which cuts in the fragment from the tubulin locus, the pTSA-11.17PRO construct was electroporated into procyclic forms of the EATRO 1125 stock of *T. brucei*. Normally these cells do not contain the AnTat

11.17 gene [20]. A control construct, identical except for the absence of the VSG gene, was electroporated in parallel (pTSA-C). A densitometric analysis of the data shown in Figure 1(A) indicated that, in the case of pTSA-11.17PRO, five tandem copies of the plasmid were integrated since, in addition to the single flanking fragments (arrowed 6.2 kb and 5 kb *Bam*HI fragments 5' and 3' respectively to the plasmid), four copies of the whole construct were detected (7.6 kb *Bam*HI fragment; ●). This analysis showed that, in the case of pTSA-C, a single copy of the plasmid was integrated. As expected, the AnTat 11.17 VSG mRNA was only synthesized in the transformed cells containing the AnTat 11.17 gene (Figure 1B). No differences in the amounts of actin or procyclin mRNAs were observed between trypanosomes transformed with pTSA-11.17PRO and pTSA-C (Figure 1B), whereas three to five times more HYG^R mRNA was present in pTSA-11.17PRO than in pTSA-C (results not shown). The size of the AnTat 11.17 mRNA (about 1.9 kb) was compatible with that predicted from the strategy of gene construction, assuming that the splice and polyadenylation sites of the procyclin UTRs were used. This was confirmed by cDNA analysis (results not shown).

Synthesis of VSG and procyclin

In contrast with control pTSA-C procyclic cells, the procyclic cells transformed with pTSA-11.17PRO express proteins that react with the polyclonal antibody anti-(AnTat 11.17 VSG) on Western blots (Figure 2). The level of VSG expressed by the transformed procyclics is lower than that expressed by AnTat 11.17 bloodstream forms (approx. 20-fold lower per cell, see

Figure 4 Immunodetection of the VSG on the surface of procyclic forms transformed with either pTSA-C or pTSA-11.17PRO

(A)–(D) Immunofluorescent staining of formaldehyde-fixed cells using rabbit anti-(AnTat 11.17 VSG) antibodies (A, C, infection serum; B, D, hyperimmune serum) followed by FITC-conjugated donkey anti-rabbit antibodies. (a) to (d) The same cells using phase-contrast microscopy ($\times 35875$).

Figure 5 Electron-microscopic immunolabelling of VSG and procyclin

Procyclic forms transformed with either pTSA-11.17PRO (a, b, c) or pTSA-C (d, e) were immunolabelled with a mixture of anti-VSG and anti-procyclin antibodies, whose secondary antibodies were respectively conjugated to 5 nM (arrows) and 10 nM (arrowheads) gold particles; f, flagellum; bar = 0.1 μ m.

Figure 2 middle panel). The transformed cells also appear to express slightly less procyclin than the control procyclics (Figure 2, bottom panel).

Phase separation of a Triton X-114 lysate of the transformed cells (Figure 3) showed that a 55 kDa species that reacts with anti-(AnTat 11.17 VSG) antibodies partitioned predominantly into the detergent phase. The apparent molecular mass and phase partition behaviour of this band suggest that it is the intact amphiphilic membrane-form VSG. The lower-molecular-mass bands were mostly hydrophilic and partitioned into the aqueous phase. The lower-molecular-mass bands were also found in the culture medium supernatant, suggesting that they are secreted from the cells (results not shown). The nature of the minor hydrophilic 56 kDa species is not known, but it could represent nascent VSG without a GPI anchor.

VSG localization

The location of the VSG in the procyclic cells transformed with pTSA-11.17PRO was determined by immunofluorescence using either an infection serum raised against live AnTat 11.17 bloodstream forms, or a hyperimmune serum raised against the purified AnTat 11.17 VSG. The data in Figure 4 indicate that both types of antibody were able to stain the entire surface of the transformed cells, whereas the controls did not show any reaction.

Figure 6 Comparison of the VSG anchor in the procyclic pTSA-11.17PRO transformants of *T. brucei* and the bloodstream AnTat 11.17 clone of *T. gambiense*

Proteins soluble in Triton X-114 were treated with PI-PLC and repartitioned in Triton X-114. Aqueous (aq) and detergent (det) phases were analysed by Western blotting after SDS/PAGE (10% gel) and probing with anti-(AnTat 11.17 VSG) antibodies.

Both types of cell were also stained by anti-procyclin antibodies (results not shown).

Electron-microscope immunolabelling was performed to refine the detection of the VSG and procyclin in the transformed procyclic forms. The trypanosomes were incubated with a mixture of anti-(AnTat 11.17 VSG) rabbit antibodies and anti-EP-procyclin mouse IgGs followed by anti-rabbit and anti-mouse antibodies conjugated with 5 and 10 nM gold particles respectively. As shown in Figures 5(a)–5(c), both antigens were found to be present over the surface of cells transformed with pTSA-11.17PRO, whereas the VSG was not detected on the control cells (Figures 5d and 5e). In accordance with the results obtained from Western blots (Figure 2), the labelling with 10 nm gold particles (anti-procyclin) was significantly reduced in pTSA-11.17PRO transformants compared with the control pTSA-C cells. In contrast with the control, the labelling of procyclin in the VSG-expressing cells appeared to be very poor in some places, such as on the flagellar sections shown in Figures 5(a) and 5(c). No labelling was observed when the two primary antibodies were omitted from the incubation medium.

Nature of the VSG anchor

The membrane-form VSGs from AnTat 11.17 bloodstream forms and from the pTSA-11.17PRO procyclic transformants were obtained in the detergent phases of Triton X-114 phase separations and treated with and without bacterial PI-PLC followed by a further round of Triton X-114 phase separation (Figure 6). As expected, the GPI-anchored bloodstream-form VSG was found in the detergent phase before PI-PLC treatment and in the aqueous phase after PI-PLC treatment (Figure 6, upper panel).

Figure 7 [^3H]Ethanolamine labelling of VSG expressed in the procyclic pTSA-11.17PRO transformants of *T. brucei*

(A) Whole-cell lysates of cells transformed with pTSA-C (lane 1) and with pTSA-11.17PRO (lane 2). [^3H]Ethanolamine-labelled whole-cell lysates were analysed by SDS/PAGE (10% gel) and fluorography after impregnation of the gels with En^3Hance . The putative VSG band is indicated by the arrow. (B) A whole-cell lysate of [^3H]ethanolamine-labelled cells transformed with pTSA-11.17PRO (lane 1) was immunoprecipitated with preimmune antibodies (lane 2) or anti-(AnTat 11.17 VSG) hyperimmune antibodies (lane 3). The arrow indicates the VSG band. The positions of EP-procycillin and GPEET-procycillin are indicated on the left of the fluorographs, and the positions of prestained molecular-mass markers are indicated on the right.

In contrast, the VSG expressed in the procyclic cells was resistant to PI-PLC and remained in the detergent phase (Figure 6, lower panel). The PI-PLC-resistance of the VSG expressed in the procyclic cells can be interpreted as follows: (i) the VSG could contain a PI-PLC-resistant GPI anchor, typical of GPI anchors with a fatty acyl chain attached to the inositol ring [35] and/or (ii) the VSG could have failed to receive a GPI anchor and retained its hydrophobic C-terminal GPI-anchor-addition signal peptide. In order to distinguish between these possibilities, (a) the transformed procyclic cells were labelled with [^3H]ethanolamine (a component of all GPI anchors), and (b) purified VSG was subjected to human serum GPI-PLD (an enzyme that cleaves both PI-PLC-sensitive and PI-PLC-resistant GPI anchors).

(a) Transformed procyclic cells (pTSA-11.17PRO) and the control cells (pTSA-C) were labelled with [^3H]ethanolamine and the whole-cell lysates were analysed by SDS/PAGE and fluorography (Figure 7A). The most prominent [^3H]ethanolamine-labelled band in the whole-cell lysates is most probably elongation factor 1α , a ubiquitous 53 kDa eukaryotic protein containing amide-linked ethanolamine attached to glutamic acid [36]. The diffuse [^3H]ethanolamine-labelled material running below this band corresponds to EP-procycillin (Figure 7A) and EP-procycillin and GPEET-procycillin (Figure 7B). Variability in the relative levels of EP- and GPEET-procycilins, according to the age of the cultures, has been previously noted [34]. The minor band present only in the VSG-expressing cells indicated with an arrow (Figure 7A) had the apparent molecular mass (55 kDa) of VSG and could be selectively immunoprecipitated with anti-(AnTat 11.17 VSG) antibodies (Figure 7B). These results suggest that at least some of the VSG expressed in the transformed procyclics contains a GPI anchor. However, because the VSG anchor was resistant to PI-PLC, it was important to assess the proportion of VSG that contained a GPI anchor by performing a GPI-PLD digest.

(b) To analyse the GPI-PLD susceptibility of the 55-kDa VSG expressed in procyclic transformants, cells were lysed and extracted with Triton X-114. After phase separation, the detergent phase, containing the amphiphilic membrane-form VSG, was applied to an octyl-Sepharose column and eluted using a propan-1-ol gradient. The VSG and endogenous procycillin were eluted as overlapping peaks between 20 and 30% of propan-1-

ol. The octyl-Sepharose-purified VSG was mixed with an aliquot of purified *T. brucei* GPEET-procycillin (which contains a GPEET repeat domain [34]), treated with and without GPI-PLD and subjected to wide-pore reverse-phase HPLC. The elution positions of the VSG and GPEET-procycillin were determined by dot-blot analysis of the column fractions with anti-(AnTat 11.17 VSG) and anti-GPEET-procycillin antibodies respectively. The added GPEET-procycillin, with a fully characterized PI-PLC-resistant 2-*O*-acyl(*sn*-1-*O*-stearyl-2-lyso-PI) GPI anchor [34], acted as an internal control for GPI-PLD activity. The successful cleavage of procycillin by GPI-PLD could be judged by the shift in the elution position of the procycillin on reverse-phase HPLC, implying a reduction in hydrophobicity as a result of the removal of *sn*-1-*O*-stearylphosphatidic acid (Figures 8A and 8B). Under these conditions, the VSG was also quantitatively shifted by a similar amount on the reverse-phase HPLC column (Figures 8C and 8D), suggesting that essentially all of the VSG contains a GPI membrane anchor.

Taken together, the data described above show that the VSG that partitions into Triton X-114 represents material that is processed to a GPI-anchored form and that the lower-molecular-mass hydrophilic material that reacts with anti-VSG antibodies probably consists of VSG-degradation products.

Procycillin is known to contain a large sialylated branched polyactosamine side chain attached to the GPI anchor glycan [8] which can be substantially degraded using a mixture of *A. ureafaciens* sialidase, bovine testes β -galactosidase and jack-bean β -hexosaminidase [34]. This treatment was performed on a sample of GPEET-procycillin, which showed the expected reduction in apparent-molecular-mass SDS/PAGE and Stains-All staining (results not shown). An identical treatment of a whole-cell lysate of the transformed procyclics was performed and the products were analysed by SDS/PAGE and Western blot with anti-(AnTat 11.17 VSG) antibody (Figure 9). The treated sample showed a reduction in apparent molecular mass and a significant reduction in heterogeneity compared with the mock-treated sample, suggesting that the VSG GPI anchor contains a similar modification to that found on procycillin, although probably less extensive judging by the relatively small reduction in apparent molecular mass. Almost no shift in the VSG band was observed when the sialidase was omitted from the exoglycosidase mixture (results not shown), consistently with the notion that all or most of the β -galactosidase/ β -hexosaminidase-sensitive side chains are capped with sialic acid. Shifts in the apparent molecular mass of small amounts of the VSG fragments at 45 and 35 kDa were also observed (Figure 9). However, since these fragments do not partition into Triton X-114 (see Figure 3), we may assume that they do not contain a GPI anchor and that the additional bands arise from proteolysis.

Nature of the VSG N-linked oligosaccharide

The whole-cell lysate was treated with endoglycosidase H and with PNGase F before SDS/PAGE and Western blotting with anti-(AnTat 11.17 VSG) antibody (Figure 10). The endoglycosidase H- and PNGase F-sensitivities of the VSG band demonstrate that it is N-glycosylated. Interestingly, the main VSG band shifts upwards on PNGase F digestion whereas it shifts downwards on endoglycosidase H digestion. However, treatment with PNGase-F after endoglycosidase H did not result in any further molecular-mass shift (results not shown), suggesting that the aberrant upward shift of this glycoprotein is due to the Asn to Asp conversion produced by PNGase F. These results show that the single N-glycosylation site of VSG 11.17 (at

Figure 8 Reverse-phase HPLC analysis of procyclin and VSG before and after GPI-PLD treatment

Octyl-Sepharose-purified VSG was mixed with an aliquot of purified GPEET-procyclin, treated without (**A** and **C**) and with (**B** and **D**) GPI-PLD and subjected to wide-pore reverse-phase HPLC. The 80 HPLC column fractions were concentrated and subjected to dot-blot analysis with anti GPEET-procyclin (**A** and **B**) and anti-(AnTat 11.17 VSG) (**C** and **D**) antibodies. Row A corresponds to fractions 1–12, row B to fractions 13–24 etc.

Figure 9 Western-blot analysis of proteins from transformed procyclics before and after treatment with mixed exoglycosidases

Whole-cell lysates of cells transformed with pTSA-11.17PRO were treated with (+) and without (–) a mixture of sialidase, β -galactosidase and β -hexosaminidase and analysed by SDS/PAGE (10% gel) followed by Western blotting and staining with anti-(AnTat 11.17 VSG) antibody.

Figure 10 Western-blot analysis of proteins from transformed procyclics before and after endoglycosidase treatment

Whole-cell lysates of cells transformed with pTSA-11.17PRO were treated with (Endo H + and PNGase F +) or without (Endo H – and PNGase F –) the endoglycosidases. The products were analysed by SDS–PAGE (10% gel) followed by Western blotting and staining with anti-(AnTat 11.17 VSG) antibody.

Asn-368) is occupied with simple N-linked oligomannose structures.

DISCUSSION

The genomic targeting of a plasmid construct containing the AnTat 11.17 VSG gene of *T. gambiense* allowed the expression of this VSG in cultured procyclic forms of *T. brucei*. The VSG is produced in reasonable amounts, probably about 5×10^5 molecules per cell. The antigen is correctly routed to the exterior surface of the entire plasma membrane, and it appears to present the epitopes normally found on the surface of AnTat 11.17 bloodstream forms. The only anomaly detected is the presence of several minor AnTat 11.17-specific peptides of incomplete size, which are probably VSG-degradation products (see below).

In procyclic forms, the AnTat 11.17 VSG is interspersed with procyclin at the cell surface. Such an arrangement is also observed transiently during the differentiation from bloodstream to procyclic forms [1]. It would appear that the presence of the VSG

leads to a significant reduction in the amount of procyclin. This effect is not observed at the level of transcription or RNA processing, as the amount of procyclin mRNA is identical in both control and VSG-expressing procyclic forms. Therefore it must be ascribed to either a reduction in protein synthesis or an increase in protein degradation. It is tempting to speculate that the presence of large amounts of VSG mRNA leads to competition with procyclin mRNA for the same fraction of membrane-bound polyribosomes [37].

The partitioning of the full-length VSG into the detergent phase after phase separation with Triton X-114, as well as its correct presentation on the membrane surface, shows that this VSG is expressed in an amphiphilic form. The susceptibility of the membrane anchor to cleavage by GPI-PLD, and the incorporation of [³H]ethanolamine, clearly imply the presence of a GPI anchor. The resistance of this anchor to PI-PLC indicates the presence of a fatty acid in hydroxyester linkage to the inositol ring [35]. Thus the VSG expressed in procyclics appears to contain a GPI anchor with a lipid structure typical of procyclin [6] and typical of the major GPI anchor precursor (glycolipid PP1) present in procyclic forms [10]. This result suggests, not surprisingly, that the nature of the GPI lipid moiety attached to a protein is dependent on the available GPI anchor precursor species in the parent cells. The sensitivity of the VSG band to digestion with a mixture of sialidase, β -galactosidase and β -hexosaminidase suggests that, like procyclin, the VSG GPI anchor acquires a sialylated branched polylactosamine-containing side chain. Therefore we may conclude that different proteins expressed in procyclic trypanosomes receive not only the same GPI anchor precursor but also similar GPI glycan modifications.

The VSG expressed in procyclics is also N-glycosylated with endoglycosidase H-sensitive oligomannose oligosaccharides. However, like procyclin (N. Zitzmann, unpublished work), type-1 bloodstream-form VSGs (like VSG AnTat 11.17) normally exclusively express oligomannose N-linked oligosaccharides. Therefore we cannot conclude from this particular example whether or not the N-glycosylation of VSGs in general, many of which are processed to express complex N-linked oligosaccharides [38], is influenced by expression in procyclics.

The presence of substantial amounts of degraded VSG in the transformed procyclics, and in the culture medium, suggests that VSG folding and/or processing is less efficient in procyclics than in bloodstream-form trypanosomes. The former possibility might reflect the lower temperature at which procyclic cells are cultured (27 °C rather than 37 °C). With regard to the latter possibility, there is an interesting precedent for the inefficient processing of GPI-anchored proteins when expressed in a different cell type [14]. In that study, Moran and Caras showed that the C-terminal GPI-addition signal peptide of a *T. brucei* VSG functioned relatively poorly in mammalian COS cells and ascribed this phenomenon to the relatively large size of the amino acid triplet at the VSG signal peptide cleavage/GPI attachment site compared with comparable mammalian sequences. The degradation of the non-GPI-anchored VSG seen in this study might be similar to that observed in COS cells [14], except that in COS cells mis-processed and degraded protein fragments are not secreted [39]. It is interesting to note that the amino acid triplet at the cleavage/GPI attachment site for VSG 11.17 (Asp-Ser-Ser) is quite large [20] whereas that for the endogenous procyclic procyclin is much smaller (Gly-Ala-Ala) [40]. Thus it is conceivable that the transamidase that exchanges the C-terminal peptide for the GPI precursor is different in the bloodstream forms and procyclic forms of the same parasite, leading to relatively poor processing of bloodstream-form proteins expressed in procyclics.

This work gives rise to several perspectives. First, it will be interesting to analyse the effects of the simultaneous expression of two different VSGs on the surface of bloodstream forms obtained by cyclical transmission of the VSG-expressing procyclics. One of the VSGs, AnTat 11.17, is expected to be constitutive, whereas the other should be variable. However, in order to perform this experiment, the UTRs of the VSG mRNA should be compatible with expression in both bloodstream and procyclic forms, such as is the case for the UTRs of tubulin and actin genes. Second, our data indicate that procyclic forms of *T. brucei* may be used to present antigens specific to the bloodstream form of human pathogenic trypanosomes such as *T. gambiense*. Since the VSG repertoires of this subspecies appear to be more limited and more conserved than those of other subspecies of the *T. brucei* group [16–19], it is possible that the injection of procyclic forms expressing the entire metacyclic VSG repertoire of *T. gambiense* would confer some immune protection against this parasite.

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