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Regulation of surface coat exchange by differentiating African trypanosomes

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Abstract

African trypanosomes (*Trypanosoma brucei*) have a digenetic lifecycle that alternates between the mammalian bloodstream and the tsetse fly vector. In the bloodstream, replicating long slender parasites transform into non-dividing short stumpy forms. Upon transmission into the fly midgut, short stumpy cells differentiate into actively dividing procyclics. A hallmark of this process is the replacement of the bloodstream-stage surface coat composed of variant surface glycoprotein (VSG) with a new coat composed of procyclin. Pre-existing VSG is shed by a zinc metalloprotease activity (MSP-B) and glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC). We now provide a detailed analysis of the coordinate and inverse regulation of these activities during synchronous differentiation. MSP-B mRNA and protein levels are upregulated during differentiation at the same time as proteolysis whereas GPI-PLC levels decrease. When transcription or translation is inhibited, VSG release is incomplete and a substantial amount of protein stays cell-associated. Both modes of release are still evident under these conditions, but GPI hydrolysis plays a quantitatively minor role during normal differentiation. Nevertheless, GPI biosynthesis shifts early in differentiation from a GPI-PLC sensitive structure to a resistant procyclic-type anchor. Translation inhibition also results in a marked increase in the mRNA levels of both MSP-B and GPI-PLC, consistent with negative regulation by labile protein factors. The relegation of short stumpy surface GPI-PLC to a secondary role in differentiation suggests that it may play a more important role as a virulence factor within the mammalian host.

Keywords: Trypanosome; Differentiation; Variant surface glycoprotein; Procyclin; Glycosylphosphatidylinositol-specific phospholipase C; Metalloprotease

1. Introduction

African trypanosomes are digenetic parasites whose lifecycle alternates between the midgut of the tsetse fly vector and the bloodstream of mammalian hosts. Native and recently isolated stocks of trypanosomes are pleomorphic in the mammalian bloodstream, transforming from a replicating long slender form into a non-dividing short stumpy form pre-adapted for transmission into the tsetse fly. Once in the fly midgut, the short stumpy form differentiates into a replicating procyclic form. Although laboratory-adapted monomorphic strains are also capable of differentiating into procyclics in vitro [1], it is most likely that the short stumpy form is responsible for natural transmission to the fly in vivo [2–4].

The most prominent marker for differentiation of bloodstream parasites into procyclic forms is the exchange of the main surface antigens: variant surface glycoprotein (VSG) in the

Abbreviations: GPI, glycosylphosphatidylinositol; GPI-PLC, glycosylphosphatidylinositol-specific phospholipase C; HRP, horseradish peroxidase; HSP70, heat shock protein 70 kDa; MSP, major surface protease; PAS, protein A-Sepharose; PBS, phosphate buffered saline; PIC, protease inhibitor cocktail; RIPA, radioimmune precipitation assay; RNAi, RNA interference; sVSG, soluble VSG; VSG, variant surface glycoprotein

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bloodstream stage [5] and procyclin in the procyclic stage [6]. Both proteins are attached to the cell surface of their respective lifecycle stages by a glycosylphosphatidylinositol (GPI)-anchor [7]. VSG, a homodimer constituting ∼10% of total cell protein in the bloodstream stage, enshrouds the cell and forms a dense monolayer impenetrable to host serum macromolecules, e.g. host immunoglobulins. Up to 1000 VSG genes encode a superfamily of potential coat proteins, but only one is expressed at a time. It is through the regulated expression of distinct VSG genes, or antigenic variation, that the parasite is able to evade the host immune response [8]. In contrast to VSG, procyclin genes encode a more restricted family composed of two basic isoforms, EP and GPEET, defined by the amino acid sequences of C-terminal repeat domains [6]. Procyclin is also very abundant and, because its repeat domains are protease resistant, is believed to provide a protective glycocalyx in the hydrolytic environment of the tsetse fly midgut.

Differentiation of bloodstream stage cells and subsequent coat exchange can be induced in vitro by the addition of *cis*-aconitate to the culture media along with a temperature reduction from 37 °C to 27 °C [9]. This process is synchronous when initiated with a predominantly short stumpy population and consequently, has been shown to consist of a series of temporally regulated events [2,3,10]. Almost immediately, VSG synthesis is repressed and procyclin expression is induced. By 12 h, surface coat exchange is complete and differentiating trypanosomes enter into their first cell cycle as fully transformed procyclics.

Because differentiating cells are non-dividing, the pre-existing VSG coat cannot be eliminated by dilution and is actively removed from the cell surface by two demonstrated modes, GPI hydrolysis and endoproteolysis [11–13]. GPI hydrolysis is mediated by an endogenous GPI-specific phospholipase C (GPI-PLC) found exclusively in the bloodstream stage of the parasite [14,15]. GPI-PLC has been shown to localize to the cytoplasmic face of intracellular vesicles [16], but can also be detected on the surface of short stumpy trypanosomes [13]. Furthermore, VSG GPI-anchor hydrolysis is present at the very beginning of the differentiation process in the starting short stumpy population. The other mode of release, endoproteolysis, is mediated by a zinc metalloprotease activity that is upregulated during differentiation [13]. Fully differentiated procyclic trypanosomes also possess a robust cell surface zinc metalloprotease activity capable of releasing transgenic VSGs [17,18]. Selective metalloprotease inhibitors are capable of blocking proteolytic VSG release from both transgenic procyclics and differentiating bloodstream forms suggesting the same or similar enzymes mediate both processes [13,18]. A family of zinc metalloprotease genes related to the well-characterized major surface protease (*MSP*) genes of *Leishmania* has been discovered in African trypanosomes by genomic sequencing [19]. Based on sequence and developmental expression, *T. brucei MSP* genes can be placed into three distinct classes: *MSP-A*, *-B* and *-C*. While MSP-A and MSP-C mRNAs are exclusively expressed in bloodstream stage trypanosomes, MSP-B mRNA is more abundant in procyclics than bloodstream stage cells [20]. Moreover, RNAi analysis indicates that MSP-B activity is responsible for release of VSG from transgenic procyclics [20]. Thus MSP-B has the characteristics required for proteolytic release of VSG during differentiation, and indeed, ablation of expression reduces release of VSG in differentiating monomorphic bloodstream trypanosomes (John Donelson, personal communication). Several factors must come into play for effective coat remodelling during short stumpy to procyclic differentiation. Short-stumpy parasites are 'primed' by activation of GPI-PLC prior to initiation of differentiation, but once differentiation begins GPI-PLC must be downregulated while MSP-B activity is upregulated. In addition, to prevent loss of the newly synthesized procyclin coat the structure of the GPI anchor precursor that is attached to surface proteins must change from that found in bloodstream cells, which is susceptible to GPI-PLC activity [21,22], to that found in procyclic parasites, which is resistant [23].

In this work, we evaluate the relative contribution of the two modes of VSG release, GPI hydrolysis and endoproteolysis, as well as the precise timing of the shift to synthesis of a procyclic-type GPI anchor. We find that GPI hydrolysis plays only a supporting role in VSG release. Nevertheless, GPI synthesis switches early in the differentiation process. We also examine the interplay of these processes using inhibitors of transcription and translation to modulate MSP-B and GPI-PLC expression during the synchronous differentiation of short stumpy cells into procyclic forms. Our results suggest that MSP-B and GPI-PLC expression are coordinately and inversely regulated, most likely under the negative control of labile trans-acting factors.

2. Materials and methods

2.1. Compounds and trypanosomes

Bathophenanthroline, actinomycin D and cycloheximide were obtained from Sigma (St. Louis, MO) and dissolved as 100× stocks in dimethyl sulfoxide or water as appropriate. A pleomorphic cell line used in the differentiation assay, *Trypanosoma brucei brucei* (AnTat 1.1), was grown in Swiss Webster mice immunosuppressed with cyclophosphamide (300 mg/kg, Sigma, St. Louis, MO) at the time of infection. Cells were isolated from infected mice on day 8 when short stumpy populations were in excess of 90% as judged by morphology.

2.2. Antibodies and immunoblotting

Rabbit antiserum against *Tb*HSP70 (gene 4), AnTat 1.1 VSG and the GPI cross-reacting determinant (CRD) has been described previously [13,24]. Anti-EP procyclin antibody (monoclonal 247) was obtained from Cedarlane Laboratories Ltd. (Ont., Canada). Anti-GPEET procyclin antibody (K1 polyclonal rabbit anti-$(GPEET)_3$ peptide) was a generous gift from Dr. Peter Bütikofer (University of Bern, Switzerland). Rabbit anti-GPI-PLC was a generous gift from Dr. Mark Carrington (Cambridge University, United Kingdom). Affinity purified rabbit anti-MSP-B antibody was a generous gift from Dr. John Donelson (University of Iowa).

Cell lysis conditions and detection of GPEET procyclin by immunoblotting have been described [13,18]. For detection of AnTat 1.1 VSG, MSP-B, GPI-PLC and BiP, whole cell lysates (10^4 cell equivalents for AnTat 1.1 VSG, 10^7 cell equivalents for MSP-B, GPI-PLC and BiP) were separated by 12% SDS-PAGE and transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P transfer membrane, Millipore Corp., Bedford, MA). Membranes were blocked in immunobuffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Tween-20, 0.01% SDS) containing 5% non-fat dry milk. After rinsing, membranes were incubated for 1 h with primary antibody diluted in immunobuffer and then washed (4×, 15 min) with immunobuffer. Blots were incubated (45 min) in secondary antibody (goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate, Kirkegaard and Perry Laboratories Inc. (KPL), Gaithersburg, MD), washed as before and specific staining was visualized on X-ray film with an enhanced chemiluminescence substrate kit (Pierce Chemical Co., Rockford, IL).

2.3. Differentiation assay

In vitro differentiation of pleomorphic AnTat 1.1 has been described previously [13]. Briefly, when populations were >90% short stumpy by visual inspection, cells were harvested from infected mice by cardiac puncture and isolated from blood treated with sodium citrate (0.2%) to prevent coagulation. After incubation at 37 °C (5% CO_2) for 1 h in HMI9 media [25], cells were washed and resuspended in 27 °C Cunningham's media at 2.5×10^6 cells/ml [26]. 3 mM each of citrate and *cis*-aconitate was added along with actinomycin D (100 μM, Sigma), cycloheximide (100 μM, Sigma) or an equivalent volume of DMSO. Cells were cultured at 27 °C and time points were taken over a 24 h period. Sampling of the 0 h time point occurred immediately prior to addition of inhibitors. Cells displayed excellent morphology and motility throughout the assay period.

At each time point, 1.5×10^6 cell equivalents (0.6 ml) were removed and separated into cell and media fractions. Trypanosomal proteins were then specifically immunoprecipitated from each fraction with appropriate antibodies and separated by SDS-PAGE. For immunoprecipitations, rabbit antisera were covalently crosslinked to Protein A-Sepharose (PAS, Amersham Biosciences, Piscataway, NJ) as previously reported [13,20]. Gels were transferred electrophoretically to membranes and immunoprecipitated proteins were detected by immunoblotting as described above.

2.4. Immunofluorescence

Differentiating bloodstream stage cells (10^7) were washed once in phosphate-buffered saline containing 2 mg/ml glucose (PBSG) and resuspended at 4×10^7 cells/ml in PBS with 5% normal goat serum (NGS). The 50 μl of cells were smeared on pre-washed slides, air dried and fixed sequentially with methanol and acetone (3 min each, −20 °C). Immunostaining of cells was completed as described previously [27,28]. Specific staining was visualized with appropriate Alexa 488- and Alexa 633-conjugated secondary reagents (Molecular Probes, Seattle, WA). Images were collected at 63× magnification on a motorized Zeiss Axioplan IIi equipped with a rear-mounted excitation filter wheel, a triple pass [DAPI (4′,6′-diamidino-2-phenylindole)-fluorescein-Texas Red) emission cube, differential interface contrast optics and a Zeiss AxioCam B&W charge-coupled device camera. Images were collected using OpenLabs 3.0 software (Improvision Inc., Lexington, MA).

2.5. Northern analysis

RNAs were prepared from the same cultures as protein samples, using a QIAquick RNEasy RNA isolation kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Approximately 2 μg of total RNA was then resolved on 1% agarose gels containing 1% formaldehyde in 1× MOPS buffer. Resolved RNAs were blotted to nylon membranes (Roche, Indianapolis, IN) and hybridized with digoxygenin-labeled (Roche) anti-sense riboprobes transcribed in vitro using either the GPI-PLC or MSP-B gene as template. Hybridizations were carried out in 50% formamide buffer at 65 °C and washed with 0.2× SSC at 65 °C. After blocking and incubation with anti-digoxygenin-conjugated horseradish peroxidase signals were detected by chemiluminescence using CDP-star as a reaction substrate (Roche).

2.6. In vitro GPI biosynthesis

Washed trypanosome membranes were prepared essentially as described by Masterson et al. [29]. Briefly, enriched short stumpy parasites were cultured for 1 h at 37 °C in HMI9 media containing 10 μg/ml tunicamycin. The time zero sample was then taken for membrane preparation and differentiation was initiated as described above. One hour prior to sampling each time point, tunicamycin (10 μg/ml) was added to equivalent aliquots of differentiating cells in order to block synthesis of *N*-glycan precursors. Membranes were likewise prepared from long slender bloodstream cells isolated after 3 days of infection and from differentiated procyclics after 3 days of culture. Membranes (5×10^8 cell equivalents/ml) were stored at −80 °C.

In vitro GPI synthesis was carried out as described by Menon et al. [30]. Membranes were washed three times in reaction buffer (50 mM Hepes, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$), resuspended at the original volume, and supplemented to 5 mM $MnCl_2$ and 1 μg/ml tunicamycin (Sigma). 1 μCi of GDP-[1-^{3}H]mannose (American Radiolabeled Chemicals, St. Louis, MO; 5–15 Ci/mmol, 70% ethanol) was air dried in fresh glass reaction tubes and then dissolved in 25 μl 5× reaction cocktail (200 mM Na-ATP, 100 mM coenzyme A, 100 mM UDP-GlcNAc in reaction buffer). Reactions were started by addition of 100 μl prepared membranes and were carried out at 37 °C for 1 h. Reactions were terminated by addition of 125 μl ice cold reaction buffer. Labeled glycolipids were extracted first with chloroform/methanol/water (10:10:3) and then with water-saturated butanol. Equivalent samples were fractionated on Silica 60 plates (Merck) with chloroform/methanol/water (10:10:2.5). Plates were visualized by phosphorimaging.

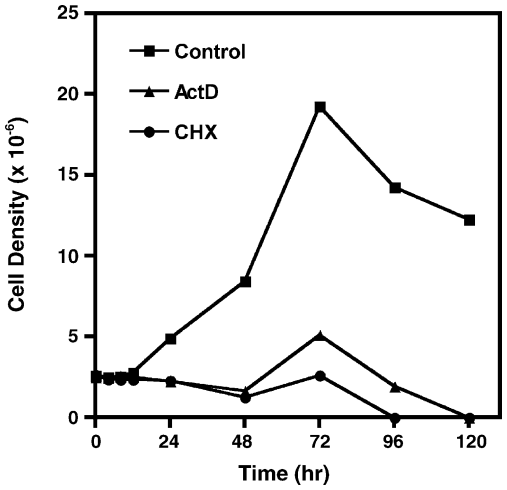

Fig. 1. Differentiation and growth of AnTat 1.1. Enriched short stumpy AnTat 1.1 bloodstream parasites were harvested from immunosuppressed mice and differentiation was induced at time zero as described in Section 2. Cells were either untreated (■, control), treated with 100 μM actinomycin D (▲, ActD), or treated with 100 μM cycloheximide (●, CHX).

3. Results[1]

3.1. In vitro differentiation of AnTat 1.1 trypanosomes in the presence of either actinomycin D or cycloheximide

We used our standard in vitro differentiation assay to investigate release of VSG from synchronously differentiating stumpy form trypanosomes [13]. Cells were isolated from immunosuppressed mice when the majority of the population (>90%) was short stumpy in morphology and differentiation was induced by a temperature shift (37–27 °C) along with the addition of citrate and *cis*-aconitate [9]. In this current work, either actinomycin D or cycloheximide was included in the assay with pleomorphic AnTat 1.1 trypanosomes to determine how VSG is released in the absence of transcription or translation, respectively. Before looking specifically at the modes of release we examined how these inhibitors affected several known hallmarks of the differentiation process. These characteristics include entrance of cells into log-phase growth, changes in both cell morphology, and surface antigen expression.

Cell density of both treated and untreated cultures was monitored over a period of 120 h (Fig. 1). Untreated AnTat 1.1 trypanosomes behaved similarly to other synchronously differentiating cell lines with logarithmic proliferation beginning at approximately 12 h post-induction [31]. In contrast, cells treated with actinomycin D or cycloheximide did not enter into log-phase growth, but remained fairly constant in cell density through the first 72 h before beginning to decline. Two further markers of differentiation, cell morphology and surface antigen expression, were investigated by performing immunofluorescence assays at 0 h and 24 h with antibodies specific to EP-procyclin and AnTat 1.1 VSG (Fig. 2). Differentiation of the control cells was evident as they transformed from short stumpy forms expressing VSG (panels A–C) into morphologically procyclic forms expressing EP-procyclin (panels D–F). Consistent with a previous study using only actinomycin D [31], treatment of cells with either inhibitor resulted in trypanosomes remaining short stumpy in morphology and retaining their VSG surface coat; there was no evidence of EP-procyclin expression (panels G–L). Nonetheless, in each inhibitor the motility and integrity of the cells remained intact as judged by microscopic examination through the 24 h time point. Overall, these results demonstrate that inhibiting transcription or translation blocks typical synchronous differentiation and cell cycle initiation as assessed by population growth and surface coat exchange.

To more closely examine the effects of actinomycin D and cycloheximide on the expression of surface coat proteins during differentiation, we used anti-GPEET-procyclin or anti-AnTat 1.1 VSG antibodies to immunoblot cell extracts taken at various time points after induction (Fig. 3). GPEET-procyclin was used to extend our analyses to an additional differentiation marker. In the absence of inhibitors, VSG levels began to decrease between 4 h and 8 h after differentiation was induced and were completely gone by 24 h (panel A, lanes 4–6). Similar to EP-procyclin, the expression of GPEET-procyclin is an early event in the differentiation process [32] and was detected as a doublet by 4 h post-induction (panel B, lane 3) that likely represents immature precursor forms [33,34]. However, unlike EP, GPEET is phosphorylated by a procyclic specific cell surface ecto-kinase [33,35,36], and the modified form of the protein was detected at the 8–12 h time points as a 25–32 kDa smear (panel B, lanes 4 and 5) [33]. Loss of the GPEET signal at the 24 h time point (panel B, lane 6) is due to lower reactivity of the anti-GPEET repeat antibody with the hyper-phosphorylated form of the protein [37].

VSG levels also decreased in the presence of actinomycin D and cycloheximide, but in each case considerable amounts remained at the 24 h time point (panel A, lanes 7–18). VSG loss was more severely affected with cycloheximide than actinomycin D as evidenced by the intensity of the VSG signals at 24 h (compare lanes 12 and 18). Expression of GPEET-procyclin was completely blocked when either inhibitor was included in the assay (panel B, lanes 7–18). Overall, these results are consistent with results seen with the immunofluorescence assay described above and confirm that inhibition of either transcription or translation disrupts the normal pattern of surface coat remodelling.

3.2. VSG release during differentiation

Both endoproteolysis by a zinc metalloprotease activity and GPI hydrolysis by endogenous GPI-PLC have been implicated in VSG release during differentiation. The two modes of release can be distinguished from each other by the electrophoretic mobility of the released VSG in the culture media: full-length soluble VSG is the product of GPI hydrolysis while truncated VSG is due to proteolysis. Using the observed appearance of these two different forms of VSG in the culture medium as our determining factor, the effects of blocking transcription or translation on VSG release during in vitro differentia-

[1] All of the results presented herein except for Figs. 3A, 5 and 8 are from the same differentiation experiment and are representative of multiple repetitions.

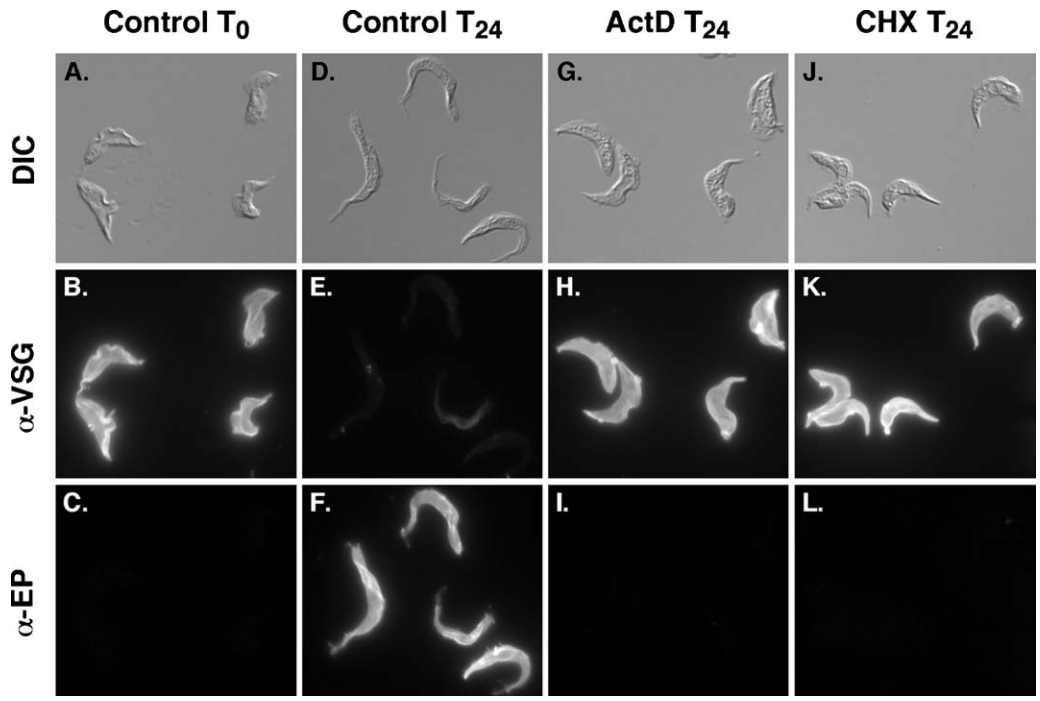

Fig. 2. Immunofluorescence analysis of differentiation. At the indicated times cells were removed from differentiation cultures containing no inhibitor, actinomycin D (ActD), or cycloheximide (CHX), and double stained for immunofluorescent imaging with anti-AnTat 1.1 VSG (panels B, E, H and K) and anti-EP procyclin (panels C, F, I and L) antibodies. Matched differential interference contrast images (DIC) were also acquired (panels A, D, G and J). All anti-VSG images and all anti-EP images were acquired at 500 ms and 125 ms, respectively, and are presented with no subsequent image processing.

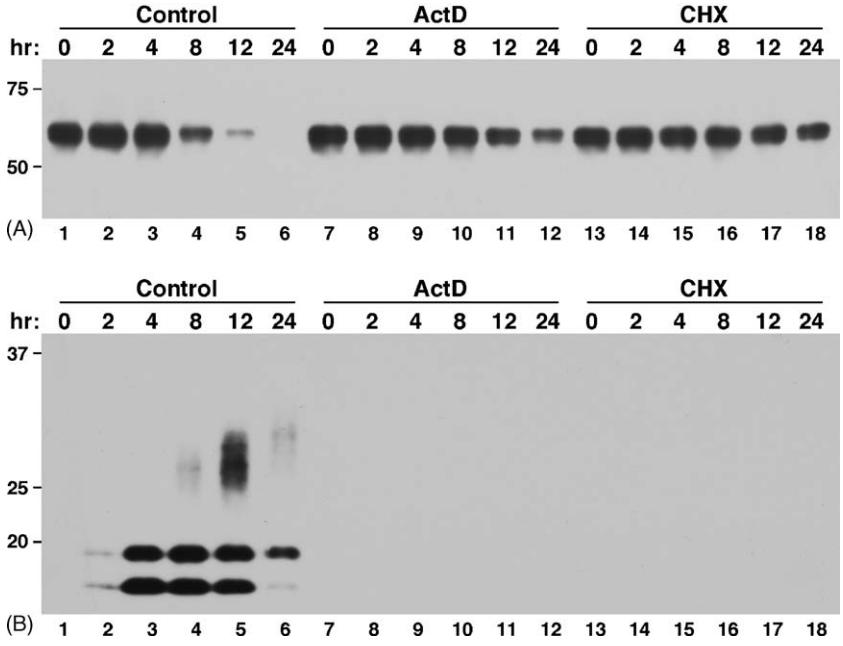

Fig. 3. VSG and GPEET procyclin expression during differentiation. Aliquots of cells were harvested at the indicated times post-induction from untreated differentiation culture (control), and cultures containing actinomycin D (ActD) or cycloheximide (CHX). Samples of each were fractionated by SDS-PAGE and immunoblotted with anti-AnTat 1.1 VSG (panel A) or anti-GPEET procyclin (panel B) antibodies. Lanes contain 10^4 (panel A) and 5×10^5 cell equivalents (panel B). Mobilities of molecular weight standards are indicated on the left in kDa.

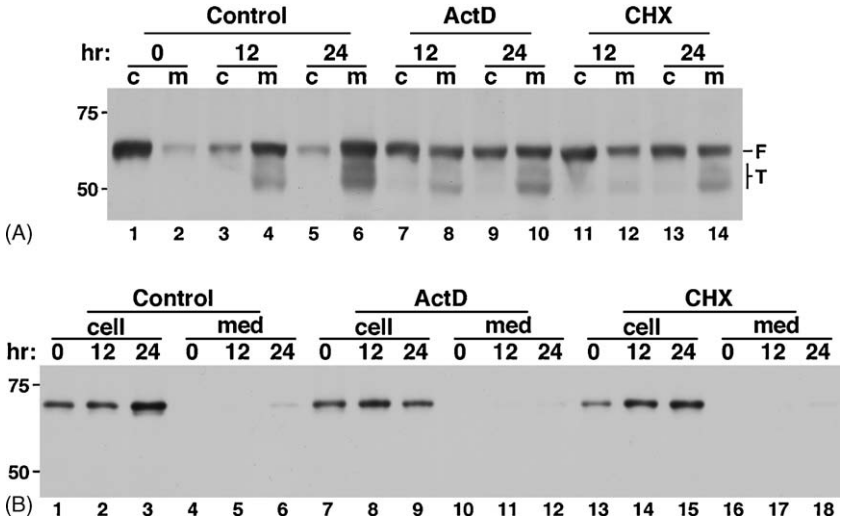

Fig. 4. Mode of VSG release during differentiation. Samples were removed from control, actinomycin-treated (ActD), and cycloheximide-treated (CHX) differentiation cultures at the indicated times and separated into cell (c) and media (m) fractions. (Panel A) VSG polypeptides were specifically immunoprecipitated, fractionated by SDS-PAGE and immunoblotted with anti-VSG. (Panel B) HSP70 polypeptides were immunoprecipitated with anti-HSP70, fractionated by SDS-PAGE, and immunoblotted with anti-HSP70. Lanes were loaded relative to original sample volume: (panel A) 2.5×10^5 cell equivalents per lane; (panel B) 5×10^5 cell equivalents per lane. Mobilities of molecular weight standards are indicated on the left in kDa. In panel A the positions of full-length VSG (F) and truncated VSG fragments (T) are indicated on the right.

tion of AnTat 1.1 trypanosomes were examined (Fig. 4). The absence of the cytosolic marker protein HSP70 from the culture medium throughout the assay period confirmed that cell integrity remained intact and therefore, any VSG release seen was not due to cell death (panel B).

All VSG was initially full-length and cell-associated at the 0 h time point (panel A, lane 1 versus lane 2). After 24 h, the majority of VSG was released into the media as a mixture of the full-length and truncated forms (panel A, lane 6). In the presence of actinomycin D, VSG release was incomplete and there was a substantial amount of cell-associated, full-length VSG remaining after 12 and 24 h (panel A, lanes 7 and 9 versus lanes 3 and 5). Furthermore, although proteolysis still occurred, the amount of truncated VSG released into the media was clearly less than the amount released in the absence of inhibitor (panel A, lanes 8 and 10 versus lanes 4 and 6). Cycloheximide had a similar, but more dramatic effect on VSG release, as truncated VSG fragments were not significantly released into the media until 24 h after differentiation was induced (panel A, lanes 11–14). These results demonstrate that endoproteolysis and GPI hydrolysis of VSG still occurred in the absence of either transcription or translation during differentiation. Note that both transcription and translation were blocked completely as there was no GPEET synthesis in the presence of either inhibitor. Thus residual VSG release is likely due to GPI-PLC and metalloprotease activities present prior to inhibitor treatment. The two modes of release are independent, with each occurring at the cell surface [13]. However, these residual activities are not sufficient to remove the entire VSG surface coat and apparently one or both need to be upregulated at the transcriptional and/or translational level during the differentiation process.

3.3. GPI-PLC and metalloprotease activity account for all VSG release during differentiation

Our results are consistent with all VSG release being mediated by the combined action of GPI-PLC and metalloprotease. However, the failure to completely release all VSG when upregulation of proteolysis is blocked suggests that pre-existing GPI-PLC is insufficient to complete coat remodelling. To further evaluate the relative contributions of the two modes of release during synchronous differentiation we subjected AnTat 1.1 trypanosomes to differentiation (Fig. 5) in the absence or presence of the zinc chelator bathophenanthroline, which we have previously demonstrated blocks proteolytic release of VSG [13]. In control cells VSG release was essentially complete by 12 h,

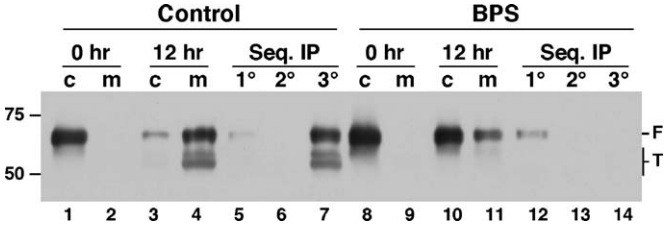

Fig. 5. Relative roles of MSP-B and GPI-PLC. Differentiation was performed with AnTat 1.1 short stumpy cells in the absence (control) or presence of 5 mM bathophenathroline (BPS) as indicated. At 0 h and 12 h cell and media fractions were analyzed as in Fig. 4. In addition, equivalent 12 h media fractions were subjected sequentially to two rounds of immunoprecipitation with saturating amounts of anti-CRD (1° and 2°), followed by anti-VSG (3°). All lanes contain 2.5×10^5 cell equivalents. Mobilities of molecular weight standards are indicated on the left in kDa. The positions of full-length VSG (F) and truncated VSG fragments (T) are indicated on the right.

and a typical profile of full-length and truncated forms was seen in the media (compare lanes 1 and 4). The proportion of VSG released by GPI hydrolysis was evaluated by sequential immunoprecipitation with anti-CRD, which specifically recognizes the protein bound portion of the hydrolyzed GPI anchor [38], followed by immunoprecipitation with anti-VSG. A single round with anti-CRD pulled down a modest amount of released VSG from the final media fraction (lane 5), and a second round with anti-CRD failed to detect additional VSG (lane 6). Subsequent immunoprecipitation with anti-VSG recovered all remaining VSG as both truncated and full-length forms, which represent metalloprotease-dependent release (lane 7). Thus the contribution of GPI-PLC to overall VSG release is relatively minor.

Bathophenanthroline completely blocked proteolytic release as evidenced by the absence of truncated fragments and the reduction of full-length VSG in the 12 h media sample (lane 11), and by the dramatic increase in cell-associated VSG (lane 10). All released VSG was recovered by a single selection with anti-CRD (lane 12), but no additional VSG was detected in further rounds of immunoprecipitation with either anti-CRD or anti-VSG (lanes 13 and 14). The complete lack of full-length VSG in the final chelated sample, as compared to the control (lane 14 versus lane 7) indicates that a significant portion of full-length VSG is actually released by proteolysis, not GPI hydrolysis as we had previously surmised [13]. Presumably cleavage occurs very close to the C-terminus, thereby eliminating the GPI anchor with little effect on overall mobility in our standard SDS-PAGE system. These results confirm that all VSG release in normal cells is dependent on the combined action of a metalloprotease, and GPI-PLC. However, despite the fact that GPI-PLC activity continues in the absence of proteolysis, GPI hydrolysis is

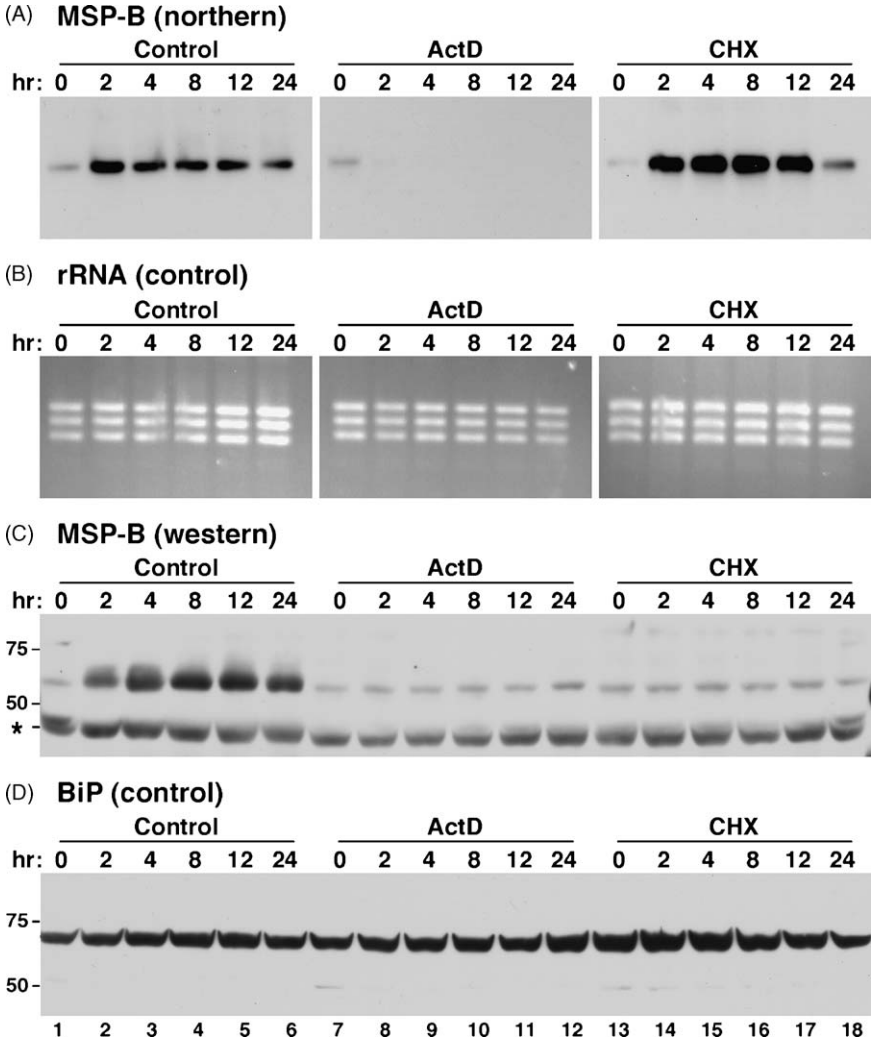

Fig. 6. Expression of MSP-B during differentiation. Samples were removed at the indicated times from control, actinomycin-treated (ActD), and cycloheximide-treated (CHX) differentiation cultures and washed cells were prepared for Northern or immunoblot analyses. (Panels A and B) Equivalent samples of total RNA (2 μg) were fractionated by electrophoresis and probed for MSP-B message (panel A). As a loading control rRNA was detected by ethidium bromide staining (panel B). In both panels A and B, all samples were run on the same gel, and all images are derived from the same exposures. (Panels C and D) Equivalent samples of cell extract were fractionated by SDS-PAGE and immunoblotted with anti-MSP-B (panel C, 10^7 cell equivalents per lane) and, after stripping, reprobed with anti-BiP antibodies (panel D). Mobilities of molecular weight standards are indicated on the left in kDa. In panel C the mobility of a non-specific cross-reactivity is indicated by the star. Detection of this band was variable in multiple experiments.

clearly insufficient to achieve full surface coat release during differentiation.

3.4. Analysis of MSP-B expression during differentiation

To investigate the expression of MSP-B metalloprotease during differentiation, Northern and immunoblot analyses were performed on samples taken at various times over a 24 h period after induction (Fig. 6). rRNA and BiP were used as loading controls for Northern and immunoblots, respectively (panels B and D). Northern blots indicate that a small amount of MSP-B mRNA is initially present at the 0 h time point (panel A, lane 1). MSP-B message levels increase at 2 h post-induction and then remain relatively constant through the 24 h time point (panel A, lanes 2–6). Actinomycin D blocks the upregulation of MSP-B mRNA and pre-existing message quickly disappears (panel A, lanes 7–12). Cycloheximide, however, resulted in increased levels of MSP-B mRNA significantly greater than control cells (panel A, lanes 13–14), suggesting an alleviation of negative regulation of this transcript. When MSP-B protein levels were assayed in the same cells, expression followed the same pattern as mRNA expression with an increase in levels observed 2 h after differentiation was induced (panel C, lanes 1–6). Both actinomycin D and cycloheximide completely blocked the upregulation of MSP-B protein expression (panel C, lanes 7–18). Altogether, these results are consistent with the observed time-dependent upregulation of proteolytic release during differentiation [13].

3.5. Expression of GPI-PLC during differentiation

GPI-PLC expression during differentiation was investigated in the same manner as MSP-B expression: Northern and immunoblot assays were performed on samples taken at various times after induction (Fig. 7). Again, rRNA and BiP were

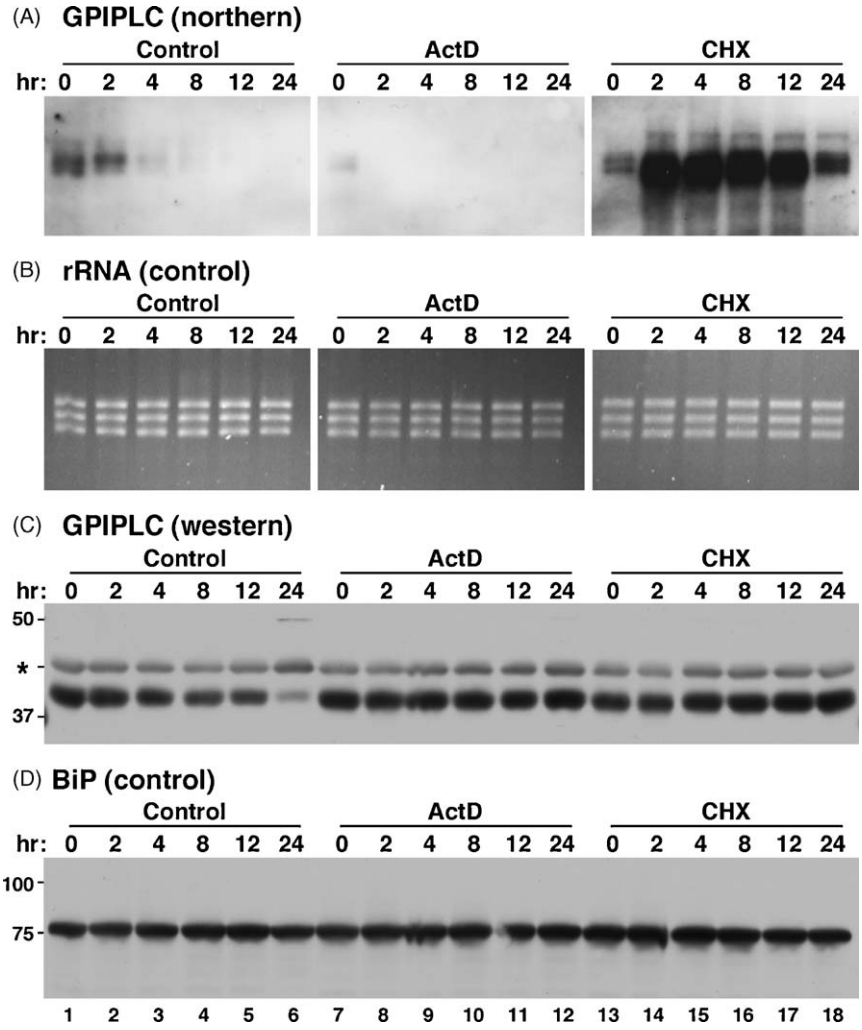

Fig. 7. Repression of GPI-PLC during differentiation. Samples were removed at the indicated times from control, actinomycin-treated (ActD), and cycloheximide-treated (CHX) differentiation cultures and washed cells were prepared for Northern or immunoblot analyses. (Panels A and B) Equivalent samples of total RNA (2 μg) were fractionated by electrophoresis and probed for GPI-PLC message (panel A). As a loading control rRNA was detected by ethidium bromide staining (panel B). In both panels A and B, all samples were run on the same gel, and all images are derived from the same exposures. (Panels C and D) Equivalent samples of cell extract were fractionated by SDS-PAGE and immunoblotted with anti-GPI-PLC (panel C, 10^7 cell equivalents per lane) or anti-BiP (panel D, 10^7 cell equivalents per lane) antibodies. Mobilities of molecular weight standards are indicated on the left in kDa. In panel C the mobility of a non-specific cross-reactivity is indicated by the star. Detection of this band was variable in multiple experiments.

used as loading controls (panels B and D). GPI-PLC mRNA is initially present at the 0 h time point, but quickly decreases and is essentially gone by 4 h post-induction (panel A, lanes 1–6). GPI-PLC protein levels, however, decrease at a slower rate and are still present in small amounts at the 24 h time point (panel C, lane 1–6). Actinomycin D and cycloheximide both inhibit the decrease observed in GPI-PLC protein levels when differentiating cells are left untreated (panel C, lanes 7–12), indicating that the enzyme is relatively stable in the absence of transcription and translation. Each inhibitor had a different effect on mRNA expression. Actinomycin D resulted in the disappearance of GPI-PLC message (panel A, lanes 7–12) while the addition of cycloheximide caused mRNA levels to increase (panel A, lanes 13–18). This elevation mimics the increase seen with MSP-B mRNA upon treatment with cycloheximide, lending support to the idea that each transcript is under negative regulation, this being alleviated by cycloheximide. These data are consistent with a recent analysis of GPI-PLC mRNA regulation in procyclic forms in the presence of protein synthesis inhibitors [39].

It is unlikely that the induction of GPI-PLC and MSP-B mRNAs is due to an unrelated activity of cycloheximide. It was previously shown that procyclin genes have increased mRNA levels in bloodstream stage parasites upon treatment with protein synthesis inhibitors [39,40]. Several structurally unrelated drugs that block different steps of translation were used, including cycloheximide, and all had the same effect. In addition, induction was shown to be gene-specific, because other constitutively expressed transcripts do not change upon cycloheximide treatment [41] (Mayho and Matthews, unpublished observations).

3.6. Developmental regulation of GPI precursor synthesis

The bloodstream stage VSG GPI anchor is PI-PLC sensitive [42], while the insect stage procyclin anchor is resistant [43,44]. This difference is reflected in the synthesis of GPI precursors in the ER [21–23,45,46]. In both stages the pathway proceeds to a complete GPI anchor with a combined diacylglycerol inositol-acyl structure (P3 in bloodstream, PP3 in procyclic). At this point the pathway diverges to generate the final diacylglycerol bloodstream (P2) and the lysoacylglycerol acylinositol procyclic (PP1) structures. These species can be distinguished by TLC mobilities and by PI-PLC sensitivity. Exactly when the shift in precursor biosynthesis occurs during differentiation is not known, and it is even possible that short stumpy cells have already shifted to a procyclic-type anchor as a pre-adaptation for differentiation. On the other hand, if the biosynthetic shift is delayed it could result in the counterproductive release of newly synthesized procyclin by GPI hydrolysis.

To investigate these issues we prepared membrane fractions from short stumpy and differentiating trypanosomes for use in in vitro GPI biosynthesis assays. As controls membranes were also prepared from dividing long slender bloodstream and fully differentiated procyclic forms. In vitro incorporation of [^{3}H]mannose from GDP-[^{3}H]mannose into defined GPI precursors was assessed by TLC (Fig. 8). Our results indicate that

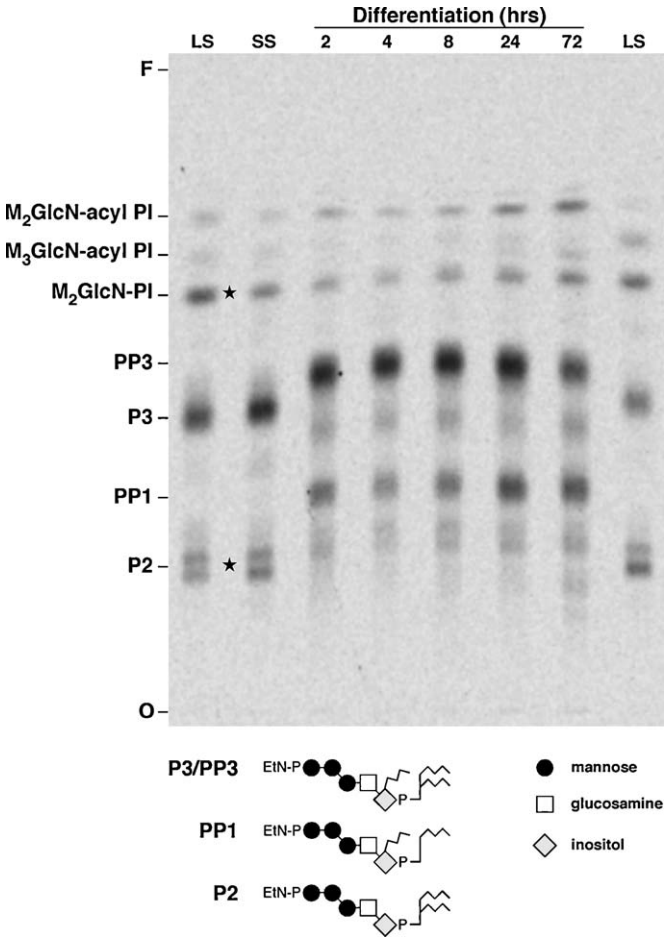

Fig. 8. GPI precursor synthesis changes during differentiation. Membranes from long slender (LS) and short stumpy (SS) bloodstream forms, from differentiating (2–24) cells, and from fully differentiated procyclic (72 h) trypanosomes were used to program in vitro GPI synthesis assays for incorporation of [^{3}H]mannose from GDP-[^{3}H]mannose into GPIs. Extracted glycolipids were fractionated by TLC and visualized by fluorography. PI-PLC sensitive glycolipid species in LS and SS extracts (data not shown) are indicated as (⋆). All other radiolabeled species were PI-PLC resistant. Identities of the various species are based on PI-PLC sensitivity and relative mobilities as described in [23,30,66]. P3 and P2 are also known as glycolipids C and A, respectively [29]. Note that the mobility difference of P3 and PP3, which have the same acyl configuration, likely reflects stage-specific differences in acyl chain lengths [66]. General structures for the P2, PP1 and P3/PP3 species are presented below. O, origin; F solvent front.

short stumpy trypanosomes synthesize a typical bloodstream set of P3 and P2 precursors. The P2 doublet likely results from partial fatty acid remodelling of the diacylglycerol moiety, the final step in bloodstream GPI synthesis [46] [complete remodelling in the in vitro assay requires addition of exogenous myristoyl-CoA, which was omitted from these reactions]. However, by the earliest time point of differentiation (2 h), GPI synthesis shifts to the procyclic pathway generating the typical procyclic precursors (PP3 and PP1). Thus, short stumpy trypanosomes are not pre-adapted for procyclic GPI synthesis. Rather, the GPI biosynthetic shift is an early event in the differentiation process thereby preventing shedding of newly synthesized procyclin.

4. Discussion

Differentiation of bloodstream trypanosomes into the procyclic form involves a series of tightly regulated developmental changes necessary for the survival of the parasite in the fly midgut. Early events resulting in the exchange of the major surface glycoproteins include repression of VSG synthesis, induction of procyclin expression, and the shedding of the old VSG coat from the surface of differentiating cells. All available data indicate that VSG shedding is accomplished by the combined action of bloodstream stage-specific GPI-PLC and a zinc metalloprotease activity that is upregulated in the procyclic stage [3,13,20,47,48]. Given its pattern of expression MSP-B is likely to play the major role in proteolytic release, but participation of other metalloprotease activities cannot be excluded at this time.

In this work we have carefully examined the coordinate and inverse expression of GPI-PLC and MSP-B during programmed differentiation in pleomorphic AnTat 1.1 trypanosomes. Consistent with a critical role in surface coat remodelling, MSP-B mRNA and protein levels are upregulated during differentiation concurrent with the onset of proteolytic release of VSG. In contrast, GPI-PLC message levels quickly decrease after differentiation is induced. Protein levels subsequently decrease, albeit at a slower rate, and dramatic loss of enzyme only occurs after the onset of cell division at 12 h (see Fig. 7C) suggesting that GPI-PLC turnover is accomplished primarily by dilution. Overall these findings agree well with earlier reports that synthesis of GPI-PLC is repressed upon differentiation leading to undetectable levels of transcript and protein in replicating log-phase procyclic trypanosomes [49–51].

Inhibition of transcription or translation derails the differentiation program; cells retain a short stumpy morphology, fail to express procyclin, and do not enter into log-phase growth. Under these conditions most VSG remains cell-associated, although small but significant amounts of VSG are still released by both GPI hydrolysis and proteolysis. As the upregulation of MSP-B protein levels is completely blocked with both types of inhibition, the low levels of pre-existing metalloprotease in bloodstream stage parasites must be responsible for the residual proteolysis. Likewise, residual GPI hydrolysis is observed since GPI-PLC is pre-activated in short stumpy cells and already present on the cell surface prior to differentiation [13]. However, when proteolysis is specifically blocked with zinc chelator, GPI-PLC is unable to complete the release of VSG, despite the persistence of significant levels of enzyme throughout the 12 h differentiation phase. To address this seeming contradiction we have examined the relative contribution of the two modes of VSG release and find, contrary to our initial model [13], that GPI hydrolysis is a minor component of VSG release during normal differentiation. One explanation for this may be that the active pool of GPI-PLC on the surface of short stumpy trypanosomes decays rapidly while loss of internal GPI-PLC occurs at a more measured pace that is dependent on the onset of cell division. Presumably in this scenario the intracellular pool is fixed during differentiation and is unable to access surface VSG. Nevertheless, despite the relatively minor role of GPI-PLC it is still possible that newly synthesized procyclin could be shed by GPI hydrolysis, as well as newly synthesized MSP-B which is predicted to be GPI-anchored based on is deduced C-terminal amino acid sequence [19]. In addition, we have recently demonstrated that a single GPI anchor of the bloodstream-type is insufficiently hydrophobic to retain monomeric GPI anchored proteins, such as procyclin, in trypanosomal membranes [52]. Consequently, it would make sense for the parasite to switch from the synthesis of the bloodstream-specific structure (dimyristoylglycerol) to the more hydrophobic and GPI-PLC resistant procyclic structure (lysoacylglycerol, acylinositol), either as pre-adapted short stump forms or early in procyclic differentiation. Our results clearly establish for the first time that short stumpy trypanosomes make a typical bloodstream-type anchor and that the switch occurs very early in the differentiation process.

An interesting outcome of our work is that inhibition of translation results in rapid super-induction of the mRNA levels of both MSP-B and GPI-PLC during differentiation. So rapid in fact that addition of inhibitor immediately *prior* to initiation of differentiation gave significant elevation of message levels in T_0 samples (Gruszynski, Matthews and Bangs, unpublished observations). These results are not totally unexpected however, as translation inhibitors have been shown to induce expression of procyclin mRNA in bloodstream stage parasites [40,41,53] and GPI-PLC in procyclic trypanosomes [39]. The simplest interpretation of these findings is that labile protein factors act as negative *trans*-regulators of specific gene expression, and such is likely the case for the super-induction we observe for both MSP-B and GPI-PLC during differentiation. Unlike higher eukaryotes, regulation of gene expression in trypanosomatids is almost exclusively post-transcriptional, primarily at the level of message stability [54]. Consequently, much attention has been focused on *cis*-acting elements in the 3′ untranslated regions of specific mRNAs that may serve as targets for *trans*-regulatory factors. Such elements have long been known in both VSG [55] and procyclin [56–58] messages, and more recently have been identified in *MSP-B* genes (John Donelson, personal communication). Furthermore, the 3′UTR of GPI-PLC is known to be sufficient for stage-regulated expression, but the length of this region (>2 kb) limits simple searches for regulatory motifs [59].

Based on these observations a simple model can be proposed for the coordinate and inverse regulation of GPI-PLC and MSP-B expression during differentiation. In the case of MSP-B, message levels are kept constitutively low in bloodstream stage parasites by a negative *trans*-acting factor. During differentiation, the ‘activity’ of the factor decreases followed by a corresponding increase in mRNA and subsequent protein to the constitutively high levels seen in procyclic parasites. When differentiating trypanosomes are treated with cycloheximide, a complete loss of the labile negative regulatory factor results in super-induction of MSP-B mRNA. A prediction of this scenario is that cycloheximide would have the same effect on MSP-B mRNA levels in normally replicating bloodstream cells. The question arises, if MSP-B is expressed in the bloodstream stage of the parasite, albeit at very low levels, why is VSG never released by endoproteolysis from dividing long slender trypanosomes? Perhaps additional modes of regulation exist for MSP-B, either at the level of translation, or by topological

sequestration from extracellular VSG. In the case of GPI-PLC, mRNA expression is relatively high in bloodstream stage cells, but is held in check by a minimal amount of a negative *trans*-factor, probably but not necessarily distinct from the factor regulating MSP-B expression. The factor increases after induction of differentiation leading to repression of GPI-PLC mRNA levels. Again, inhibition of translation results in complete loss of the factor and super-induction of GPI-PLC message during differentiation, consistent with the observation that translational inhibition induces GPI-PLC expression in dividing procyclic cells [39].

If GPI-PLC can now be relegated to a minor role in coat release during differentiation why then is it mobilized to the surface of primed short stumpy trypanosomes? Perhaps the real purpose of surface GPI-PLC is to modulate host immune responses while still in the mammalian bloodstream. As each wave of parasitemia rises in the bloodstream, transformation to the non-dividing short stumpy form occurs in a density-dependent manner [60,61], and consequently low levels of VSG will be shed into the media by GPI hydrolysis. Indeed, short stumpy parasites do shed VSG in vitro prior to induction of differentiation (Gruszynski and Bangs, unpublished observations). Furthermore, soluble VSG has been shown to induce anergy in IFN-γ stimulated host macrophage resulting in immune suppression [62,63]. Thus cell surface GPI-PLC may be viewed as a virulence factor. Consistent with this view, the primary biological phenotype for GPI-PLC null pleomorphic cell lines, which are fully capable of differentiation, is reduced virulence in in vivo mouse models [64,65], generating much lower parasitemias and prolonged host survival. It may seem counterintuitive for a parasite to deploy a virulence factor to subvert innate immune mechanisms when the result is the more rapid death of the host. But the trade-off to increased virulence is high parasitemia, with concomitant generation of short stumpy parasites, which is critical for successful transmission when the next tsetse fly takes a blood meal.

In summary, we have provided a detailed analysis of the machinery and regulation of surface coat exchange during differentiation of African trypanosomes. Our results confirm that both metalloprotease and GPI-PLC together achieve complete shedding of the old VSG coat. Critical to the remodelling process is the shift in synthesis from a bloodstream-style GPI anchor to a GPI-PLC resistant procyclic-type anchor. However, GPI-PLC can now be relegated to a secondary role in coat release. Instead, future efforts should focus on the part this enigmatic enzyme may play as a virulence factor in the mammalian host.

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