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Trypanosoma brucei: Reduction of GPI-phospholipase C protein during differentiation is dependent on replication of newly transformed cells

Sandesh Subramanya, Dora A. Armah, Kojo Mensa-Wilmot *

Department of Cellular Biology, The University of Georgia, 724 Biological Sciences, Athens, GA 30602, United States

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ABSTRACT

The protozoan parasite *Trypanosoma brucei* lives in the bloodstream of vertebrates or in a tsetse fly. Expression of a GPI-phospholipase C polypeptide (GPI-PLCp) in the parasite is restricted to the bloodstream form. Events controlling the amount of GPI-PLCp expressed during differentiation are not completely understood. Our metabolic "pulse-chase" analysis reveals that GPI-PLCp is stable in bloodstream form. However, during differentiation of bloodstream to insect stage (procyclic) *T. brucei*, translation *GPI-PLC* mRNA ceases within 8 h of initiating transformation. GPI-PLCp is not lost precipitously from newly transformed procyclic trypanosomes. Nascent procyclics contain 400-fold more GPI-PLCp than established insect stage *T. brucei*. Reduction of GPI-PLCp in early-stage procyclics is linked to parasite replication. Sixteen cell divisions are required to reduce the amount of GPI-PLCp in newly differentiated procyclics to levels present in the established procyclic. GPI-PLCp is retained in strains of *T. brucei* that fail to replicate after differentiation of the bloodstream to the procyclic form. Thus, at least two factors control levels of GPI-PLCp during differentiation of bloodstream *T. brucei*; (i) repression of *GPI-PLC* mRNA translation, and (ii) sustained replication of newly transformed procyclic *T. brucei*. These studies illustrate the importance of repeated cell divisions in controlling the steady-state amount of GPI-PLCp during differentiation of the African trypanosome.

1. Introduction

Trypanosoma brucei causes human African trypanosomiasis, and is transmitted to its vertebrate host through the bite of a tsetse fly. In natural settings, differentiation of the bloodstream form to the procyclic (insect stage) *T. brucei* is important for transmission of the parasite.

In the bloodstream, *T. brucei* is covered with a variant surface glycoprotein (VSG) whereas in a tsetse fly the parasite expresses procyclin (PARP) as the major surface protein. Differentiation of bloodstream to insect stage (procyclic) *T. brucei* (reviewed in (Fenn and Matthews, 2007) is characterized by increased procyclin expression and loss of VSG within 6 h of initiating transformation (Roditi et al., 1989). Cell division is arrested during differentiation, which is completed within 72 h.

In trypanosomatids, protein levels are controlled predominantly by post-transcriptional events, as initiation of transcription is rarely regulated in these organisms in (Clayton, 2002).

Glycosylphosphatidylinositol (GPI)-phospholipase C (*GPI-PLC*) is expressed in bloodstream form *T. brucei*. GPI-PLC is a virulence factor in pleomorphic *T. brucei* (Tasker et al., 2000; Webb et al., 1997). The enzyme stimulates endocytosis of transferrin in bloodstream *T. brucei* (Subramanya et al., 2009), and is activated by mild acid or hypotonic conditions to cleave GPI at the endoplasmic reticulum (Subramanya and Mensa-Wilmot, 2006). GPI-PLC does not have a significant role in release of variant surface glycoprotein (VSG) from differentiating bloodstream *T. brucei* (Bülow et al., 1989a,b; Gruszynski et al., 2006; Hanrahan et al., 2009; Webb et al., 1997).

In established procyclic *T. brucei*, *GPI-PLC* enzyme activity is not detectable (Bülow et al., 1989a,b; Mensa-Wilmot et al., 1990). However, full-length mRNA of the *GPI-PLC* gene is present in established procyclic *T. brucei* (Carrington et al., 1989; Mensa-Wilmot et al., 1990), although the half-life of newly synthesized pre-mRNA is relatively short (Webb et al., 2005). The basis for disappearance of GPI-PLC polypeptide (GPI-PLCp) from procyclic *T. brucei* is not known. As part of an effort to understand possible connections between steady-state level of GPI-PLCp and differentiation of bloodstream trypanosomes, we studied stability of GPI-PLCp and the kinetics of translation of *GPI-PLC* mRNA during differentiation of bloodstream to procyclic *T. brucei*.

We found, unexpectedly, that differentiation (alone) of bloodstream to the procyclic form is not sufficient to explain the differ-

Abbreviations: BSF, bloodstream form trypanosomes; GPI, glycosylphosphatidylinositol; GPI-PLC, GPI-specific phospholipase C; GPI-PLCp, polypeptide encoded by *GPI-PLC* gene, PCF, procyclic (insect stage) *T. brucei*; VSG, variant-specific surface glycoprotein.

* Corresponding author. Fax: +1 706 542 4271.
E-mail address: mensawil@uga.edu (K. Mensa-Wilmot).

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ence in magnitude of GPI-PLCp level between established and newly transformed procyclic cells. Arrest of translation of *GPI-PLC* mRNA occurs early during differentiation, in concert with a 50% loss of GPI-PLCp in newly differentiated procyclic cells. However, multiple replication cycles are needed to reduce the level of GPI-PLCp from that found in newly differentiated procyclic cells to that reported in established procyclic lines. These observations highlight the importance of cell replication in developmentally regulated expression of a trypanosome GPI-phospholipase C. Our results have implications the developmental regulation of proteins that are highly stable in bloodstream *T. brucei*.

2. Experimental procedures

2.1. Cells

Monomorphic *T. brucei* ILTat 1.3 and *T. brucei* 427, and the pleomorphic AnTat 1.1 were used. Culture-adapted bloodstream *T. brucei* 427 was a gift from Dr. C.C. Wang (University of California, San Francisco). Established procyclic *T. brucei* 427 was kindly provided by Dr. Jay Bangs (University of Wisconsin, Madison). Bloodstream form trypanosomes were harvested from infected rats and purified by DE-52 chromatography (Cross, 1975).

Mice were inoculated intraperitoneally with *T. brucei* AnTat 1.1 and simultaneously injected with cyclophosphamide (Sigma) (300 mg/kg body weight) (Gould et al., 1986). Parasite density was approximately 4×10^7–1×10^8/ml of blood between day 5 and day 6 when they were harvested.

2.2. Materials

Fetal bovine serum (FBS) was obtained from Life Technologies (Gaithersburg, MD). Citric acid and cis-aconitate were purchased from Sigma (St. Louis, MO). All protease inhibitors were from Boehringer Mannheim (Indianapolis, IN). 5-Bromo-4-chloro-indoyl phosphate (BCIP), *p*-nitroblue tetrazolium chloride (NBT), and alkaline phosphatase conjugated goat anti-rabbit IgG were purchased from BioRad (Richmond, CA). Procyclin (anti-GPEET) antibody was a gift from Dr. Isabel Roditi (Universitat Bern, Switzerland). Anti-VSG117 and anti-AnTat.1 antibodies were provided by Dr. Jay Bangs (University of Wisconsin, Madison). Anti-VSG221 (Hoek et al., 1999) was a gift from Dr. George Cross (Rockefeller University). [^{35}S]Methionine was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

2.3. Differentiation of bloodstream T. brucei in vitro

Monomorphic trypanosomes (strains 427 and ILTat1.3) were harvested from rat blood at a density of 2×10^9/ml. Following the preparation of a "buffy coat", parasites were added to transformation medium (SDM-79 containing 10% heat-inactivated FBS, and 5 mM each of citric acid and cis-aconitate (Bülow et al., 1989a,b; Cunningham, 1977) at the density indicated (see "Figure Legends"). Cultures were incubated at 27 °C, and parasites counted at 12 or 24-h intervals. An aliquot (2×10^7) of parasites was harvested at each time point, washed with phosphate buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4), pelleted (3000*g*, 5 min), and frozen at −80 °C until use.

For differentiation of AnTat 1.1 *T. brucei*, heparinized (infected) mouse blood was added to a 50% slurry of DE-52 beads prepared in bicine buffered saline (BBS) (10 ml bed vol/ml blood), following a modification by J. Bangs of a procedure developed by Overath and colleagues (Ziegelbauer et al., 1990). Briefly, tubes containing the DE-52/blood slurry were inverted continuously for 5 min, centrifuged (1500*g*, 1 min, 25 °C), and the supernatant incubated at 27 °C. (Cells adsorbed to the DE-52 beads were recovered by washing in fresh 1× BBS, and centrifuging at 1500*g* for 20 s at room temperature. This second supernatant was pooled with the supernatant from the first centrifugation, and then centrifuged at 1500*g* for 5 min to pellet the cells.) Parasites (10^8) were washed once with 1× BBS, resuspended in HMI-9 medium containing 15% FBS (Hirumi and Hirumi, 1994), and incubated at 37 °C in 5% CO_2 for 1 h before being added to 40 ml of transformation medium (SM containing 15% heat-inactivated FBS, 5 mM citric acid, and 5 mM cis-aconitate) (density of 2.5×10^6 cells/ml). Cells were incubated at 27 °C. Beginning at inoculation and at time intervals indicated 10^7 cells were harvested. After washing with PBS, cells were frozen at −80 °C until GPI-PLC enzyme assays were performed. In addition, at specified time intervals, 2×10^6 parasites were processed for immunofluorescence microscopy (see below).

2.4. Determination of GPI-phospholipase C activity

Parasites (2×10^7) were resuspended in 200 μl of hypotonic lysis buffer (10 mM sodium phosphate, 1 mM EDTA, pH 8) containing leupeptin (2.1 μM), *N*-tosyl-L-lysine chloro-methyl ketone (TLCK) (0.1 mM) and aprotinin (0.4 U) (Hereld et al., 1986; Mensa-Wilmot et al., 1994). Cells were kept on ice for 20 min. The lysate was centrifuged (14,000*g*, 4 °C, for 10 min) and the supernatant discarded. The pellet was solubilized in 200 μl of 50 mM Tris–HCl, 5 mM EDTA, 1% NP40 (AB). Five microliter of each sample was assayed for GPI-digestion activity using [^{3}H]myristate-labeled membrane form VSG (mfVSG) as substrate (Mensa-Wilmot et al., 1995). Duplicate reaction mixtures were incubated at 37 °C for 15 min, released [^{3}H]dimyristoylglycerol ([^{3}H]DMG) was extracted with water–saturated butanol, and quantitated by liquid scintillation counting. Several dilutions (in AB) of the solubilized membrane fractions were assayed in order to obtain values within the linear range (0.1–1 units) of GPI-PLC activity. Protein content of fractions was determined with bicinchoninic acid (BCA) reagent (Pierce).

2.5. SDS–PAGE and Western blotting

For immunoblotting, proteins were transferred onto Immobilon P (Millipore) using a Trans-Blot Semi-Dry cell (BioRad) (Armah and Mensa-Wilmot, 1999). Rabbit antisera against VSGs (VSG117, VSGAntat1.1 or VSG221), procyclin (anti-GPEET), and sea urchin tubulin (anti-TUB) were used at a dilution of 1:3000 for Western blotting (Mensa-Wilmot et al., 1990).

2.6. Immunofluorescence

T. brucei AnTat1.1 bloodstream (2×10^6) were pelleted at 1500*g* for 5 min and washed twice with 1× BBS. The cells were air dried on poly-L-lysine coated cover slips for 30 min at room temperature, and fixed in acetone at −20 °C for 5 min. After rinsing twice with PBS for 5 min each time, fixed cells were blocked with 1% BSA (in PBS) for 25 min. Cover slips were incubated with primary antibody in blocking buffer for 2 h. Primary antibodies were against VSG AnTat1.1 (polyclonal anti-VSG AnTat 1.1 (1:2000 dilution) (a gift from J. Bangs), and procyclin (mouse anti-GPEET antibody, CedarLane Laboratories) (1:200 dilution). Cover slips were rinsed in PBS for 5 min (three times) and treated with blocking buffer for 25 min. Cells were then incubated with the following secondary antibodies in blocking solution for 2 h in the dark; goat anti-rabbit IgG-Alexa-Fluor 488 (Molecular Probes) (1:5000) to detect anti-VSG, and goat anti-mouse IgG – Alexa Fluor 594 (Molecular Probes) (1:5000) to localize anti-procyclin antibody. Cover slips were washed thrice with PBS (5 min each time), blotted dry, mounted on slides, and visualized with a Leica microscope (DMIRBE). Images were captured using an interline chip cooled CCD camera (Orca 9545: