Synthesis of a hydrolase for the membrane-form variant surface glycoprotein is repressed during transformation of *Trypanosoma brucei*

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A membrane-bound phospholipase C-like hydrolase present in lysates of bloodstream forms of *Trypanosoma brucei* rapidly converts the membrane form of the variant surface protein to the soluble form and 1,2-dimyristoylglycerol [(1985) M.A.J. Ferguson et al. J.Biol.Chem., 260, 4963–4968]. The hydrolase is inhibited by *p*-chloromercuribenzenesulfonate. The synthesis of the enzyme is rapidly repressed upon differentiation of bloodstream forms to procyclic cells and the enzyme activity declines to an undetectable level during subsequent growth of procyclic forms.

Variant surface glycoprotein *1,2-Dimyristoylglycerol* *Hydrolase* *Transformation* Trypanosoma brucei

1. INTRODUCTION

In the mammalian host, the unicellular flagellate *Trypanosoma brucei* is covered by a dense surface coat [1]. For a parasite population derived from a single cell (variant clone) this coat consists of a single glycoprotein species, designated variant specific glycoprotein (VSG). However, a variant clone always contains a small fraction of cells expressing antigenically different VSGs, such variants may in turn be selected and cloned. VSGs of different variant clones differ widely in their amino acid sequence, in particular in their N-terminus, while their carboxy-terminal sequences are related, the final amino acid residue being aspartic acid or serine, with the exception of one VSG that terminates in asparagine (review [2]). A common feature of different VSGs appears to be their mode of attachment to the cytoplasmic membrane. As demonstrated for the *T. brucei* strain 427 variant clone MiTat 1.4 (also known as variant 117) the carboxy-terminal aspartic acid is connected to ethanolamine via an amide bond which forms the link to a complex glycophospholipid composed of glucosamine (1 mol), mannose (2 mol), galactose (4 mol), phosphate (0–1 mol) and a *sn*-1,2-dimyristoyl-3-phosphoglycerol residue [3,4]. The phospholipid presumably anchors the protein in the cytoplasmic membrane. An enzymatic activity present in trypanosome lysates rapidly converts the membrane-bound form of VSG (mfVSG) to the conventionally isolated water-soluble form (sVSG, cf. [5,6]). This reaction results in the exposure of a new antigenic determinant on the sugar residues attached to the carboxy terminus. This determinant is common to most sVSGs and designated the cross-reacting (CR-) determinant.

Our interest in the enzymatic activity which converts mfVSG to sVSG stems from studies on the

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Abbreviations: VSG, variant surface glycoprotein; mfVSG, membrane-form VSG; sVSG, soluble VSG; CR-determinant, cross-reacting determinant; $DecO(EtO)_8H$, decylpolyoxyethylene ether; $ClHgBzSO_3$, *p*-chloromercuribenzenesulfonate; CCA, citrate plus *cis*-aconitate; PMSF, phenylmethylsulfonyl fluoride; TLCK, tosyl-L-lysine chloromethyl ketone; DTT, dithiothreitol

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release of the surface coat from bloodstream trypanosomes during morphological transformation to uncoated procyclic cells. Normally, this differentiation step takes place in the midgut of the tsetse fly after ingestion of trypanosomes with the blood meal. Alternatively, an essentially synchronous transformation of bloodstream forms to procyclic cells can be initiated in an in vitro culture system by a decrease in temperature from 37 to 27°C and the addition of *cis*-aconitate or a combination of *cis*-aconitate and citrate [7,8]. Transformation begins with a rapid repression of VSG synthesis and, subsequently, the surface coat is released into the medium as sVSG, part of which is degraded by proteolytic cleavage. Therefore, during transformation one or several hydrolytic activities obtain access to the surface-bound mfVSG, possibly by secretion into the flagellar pocket. The mfVSG to sVSG converting activity present in bloodstream forms is an obvious candidate to play a role in coat release during transformation. Therefore, we have monitored the level of enzymatic activity throughout the conversion of bloodstream forms to procyclic cells.

2. MATERIALS AND METHODS

Incomplete Iscove's medium was purchased from Flow Laboratories (Meckenheim, FRG, Cat.-No.10-357-22), CNBr-activated Sepharose 4B and protein A-Sepharose 6MB from Deutsche Pharmacia (Freiburg, FRG), decylpolyoxyethylene ether ($DecO(EtO)_8H$) from Imperial Chemical Institute (Wilmington, DE) and [9,10-$^3H_2$]myristic acid (58 Ci/mmol) from Amersham Buchler (Braunschweig, FRG).

2.1. *Trypanosomes*

T. brucei variant clones MiTat 1.4 (117), 1.5 (118) and 1.6 (121) were obtained from Dr G.A.M. Cross (Rockefeller University, New York), the monomorphic variant clone AnTat 1.1 was a gift of Dr D. LeRay (Institut de Medecine Tropicale, 'Prince Leopold', Antwerp, Belgium).

2.2. *Preparation of [^{3}H]myristic acid-labeled mfVSG*

MiTat 1.4-trypanosomes harvested from mouse blood were washed twice in cold Iscove's medium lacking transferrin and soybean lecithin but containing 5 mg/ml bovine albumin (Sigma, Cat. No.A7030), 5 μM adenosine, 20 μM guanosine, 10 μM thymidine, and 20 μM cytosine. After incubation of 8×10^8 cells in 20 ml of this medium supplemented with 50 μCi/ml [^{3}H]myristic acid for 3 h at 37°C, the cells were centrifuged and lysed in 8 ml PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.2), 10 mg/ml octylglucoside, 0.1 mM TLCK and 10 mM $ClHgBzSO_3$. Polyclonal anti-MiTat 1.4 sVSG antibodies were purified by ammonium-sulfate precipitation and DEAE cellulose chromatography and coupled to CNBr-activated Sepharose 4B by standard procedures. The cell lysate was centrifuged at 8000 × *g* for 20 min and the supernatant was passed over the antibody column (15 ml containing 40 mg IgG). The column was washed overnight with PBS/10 mg/ml $DecO(EtO)_8H$ and the bound mfVSG was eluted with 0.2 M glycine (pH 2.8), 10 mg/ml $DecO(EtO)_8H$. The eluant was neutralized with 1 M Tris-Cl (pH 8.0) and the radioactive fractions were pooled and dialyzed overnight against PBS, 10 mg/ml $DecO(EtO)_8H$. Analysis of the product by SDS-polyacrylamide gel electrophoresis showed only a single radioactive band characteristic for mfVSG. A typical preparation contained 35×10^3 cpm/μg mfVSG.

2.3. *Trypanosome lysate*

Freshly harvested trypanosomes of variant clone MiTat 1.4 were washed once in trypanosome dilution buffer (TDB, cf. [9]) and lysed in 50 mM Hepes, 0.5 mM DTT, 2.5 mM EDTA and 10 mg/ml $DecO(EtO)_8H$ at a density of 1×10^8 cells/ml. This extract was used directly for the estimation of mfVSG hydrolase activity (cf. legend to fig.1).

2.4. *Transformation procedure*

The transformation protocol has been detailed before [8]. Cells were either grown overnight at 37°C in medium B + 15% inactivated horse serum in the presence of 3 mM citrate + 3 mM *cis*-aconitate (CCA) and then transferred to medium SDM79 + 15% inactivated fetal calf serum (iFCS) + CCA and further incubated at 27°C or they were incubated overnight at 37°C in medium B + 15% iFCS + CCA and then transferred to

27°C without a medium change. For further details see [8,10].

2.5. *Separation of coated and uncoated trypanosomes*

8×10^7 trypanosomes were incubated in 1 ml medium B with 100 μl heat inactivated anti-sVSG antiserum at 0°C for 30 min and then passed at 4°C over a protein A-Sepharose 6 MB column (2 ml) equilibrated with the same medium. Cells not adhereing to the column were collected by centrifugation and fixed for immunofluorescence [8] or lysed as described above to measure the hydrolase activity.

3. RESULTS

3.1. *Estimation of the mfVSG hydrolase activity*

An initial enzyme assay is based on the observation that the conversion of mfVSG to sVSG leads to the exposure of the CR-determinant on sVSG [5]. Trypanosomes of variant clone AnTat 1.1 are metabolically labeled with [^{35}S]methionine and an mfVSG-containing extract is prepared by boiling the cells for 1 min in PBS/1 mM TLCK/0.1 mM PMSF/10 mg/ml octylglucoside [5]. The total VSG (mfVSG + sVSG) in the soluble fraction of such an extract amounts to 30% of the labeled proteins, as shown by immunoprecipitation with anti-AnTat 1.1-sVSG antibody. The CR-antibody prepared by affinity chromatography of an anti-MiTat 1.4-sVSG antiserum on a MiTat 1.6-sVSG Sepharose column precipitates 3.3% of the radioactivity from the same extract. As this antibody is sVSG specific it demonstrates that the AnTat 1.1-VSG in the extract contains 90% mfVSG and 10% sVSG.

Incubation of the [^{35}S]mfVSG-containing extract with lysates from a heterologous variant, i.e. MiTat 1.5, leads to the conversion of mfVSG to sVSG which can be quantitated by subsequent immunoprecipitation of sVSG by the CR-antibody. Using this assay two noteworthy observations were made. First, the enzymatic activity converting mfVSG to sVSG is associated with the total membrane fraction of trypanosome lysates, agreeing with recent results from other laboratories [4,11], and it can be solubilized in an active form by detergents such as 10 mg/ml $DecO(EtO)_8H$. Second, the activity can be inhibited by the thiol reagent *p*-chloromercuribenzenesulfonate ($ClHgBzSO_3$, 1 mM) but not by *N*-ethylmaleimide (5 mM) or iodoacetamide (5 mM). Inhibition by $ClHgBzSO_3$ can be quantitatively reversed by an excess of mercaptans. $ClHgBzSO_3$ is useful for terminating the enzymatic conversion of mfVSG to sVSG. When added during lysis of trypanosomes endogenous enzyme activity is completely inhibited. As mfVSG purified from such lysates is still able to act as a substrate in the conversion of mfVSG to sVSG, it is the enzyme rather than the substrate that interacts with the inhibitor. Finally, the presence of a reactive sulfhydryl group in the hydrolase permits the use of an organomercurial agarose affinity column (Affi-Gel 501, BioRad-Laboratories, München) as an effective step in the partial purification of the enzyme [12].

The recent demonstration that the mfVSG contains an *sn*-1,2-dimyristoyl-3-phosphoglycerol residue as a membrane anchor [4] permits the use of a much simpler assay for estimating the hydrolytic activity. mfVSG is labeled with [^{3}H]myristic acid and purified on a Sepharose column containing immobilized anti-sVSG antibody. Upon incubation of [^{3}H]myristic acid-labeled mfVSG with a detergent lysate of unlabeled trypanosomes, radioactivity is released which can be extracted by a mixture of hexane:isopropanol (3:2). Fig.1 shows the release of radioactivity extracted by organic solvents as a function of trypanosomal protein in a detergent extract. From the linear part of the curve an activity of 1 $nmol \cdot h^{-1} \cdot 10^{-8}$ trypanosomes can be calculated. This rate is at least 10-times below the maximum rate as a 10-fold increase in substrate concentration increases the rate 10-times.

As noted by Ferguson et al. [4] and confirmed here, prolonged incubation of [^{3}H]myristic acid-labeled mfVSG with a crude trypanosome extract yields free myristic acid as a product, while partially purified enzyme preparations yield 1,2-[^{3}H]dimyristin. This indicates that a crude lysate contains a phospholipase C-like activity which converts mfVSG to sVSG + 1,2-dimyristin and, in addition, one or several lipases which release myristic acid either from mfVSG directly or from the 1,2-dimyristin. The experiment depicted in fig.2 suggests that the release of 1,2-dimyristin catalyzed by the lysate proceeds at a greater rate than the formation of free myristic acid. Under

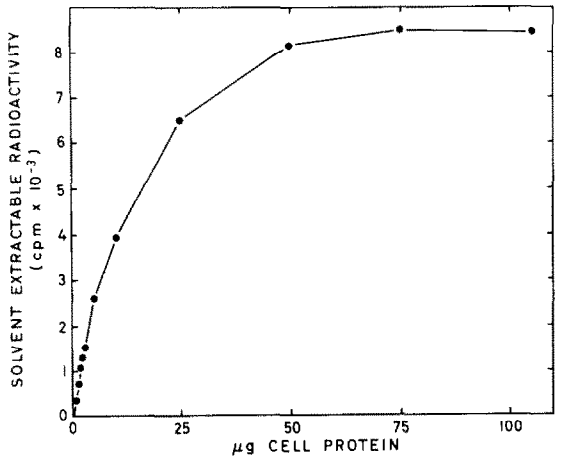

Fig.1. Cleavage of [^{3}H]myristic acid-labeled mfVSG upon incubation with a detergent lysate of trypanosomes. 0.3 μg purified [^{3}H]myristic acid-labeled mfVSG (10600 cpm) was incubated with different amounts of a detergent lysate of trypanosomes in 200 μl of 50 mM Hepes, pH 7.0, 0.5 mM DTT, 2.5 mM EDTA and 10 mg/ml DecO(EtO)$_8$H. After 30 min at room temperature the reaction was stopped by the addition of 50 μl of 20% acetic acid and the released radioactivity extracted with 500 μl hexane-isopropanol (3:2). The radioactivity in 200 μl of the organic phase (total volume about 250 μl) is plotted against the amount of trypanosomal protein added to the incubations.

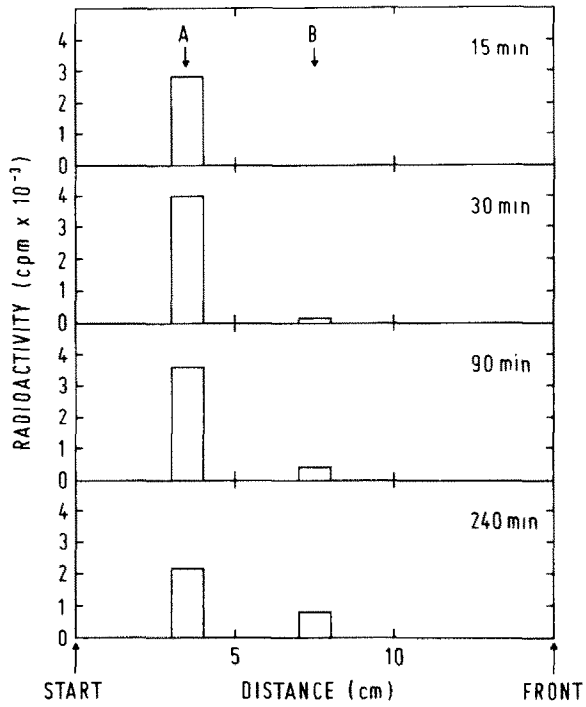

Fig.2. Analysis of the products released from [^{3}H]myristic acid-labeled mfVSG upon incubation with a detergent extract of trypanosomes. Purified [^{3}H]myristic acid-labeled mfVSG (0.3 μg, 10600 cpm) was incubated with 100 μg protein of a detergent lysate of trypanosomes in 200 μl 50 mM Hepes, pH 7.0, 0.5 mM DTT, 2.5 mM EDTA, 10 mg/ml DecO(EtO)$_8$H. At the times indicated the reaction was stopped by the addition of 50 μl of 20% acetic acid and released 1,2-dimyristin and myristic acid were recovered by hexane-isopropanol (3:2) extraction. 100 μl of the organic phase was analyzed by thin-layer chromatography on silica G gel plates using petroleum ether, diethyl ether, acetic acid (70:30:2) as a solvent. 1 cm sections of the gel were scraped off and the radioactivity estimated by liquid scintillation counting. Arrows A and B show the position of authentic 1,2-dimyristin and myristic acid, respectively.

conditions where the conversion of mfVSG to sVSG is complete, free myristic acid is hardly detectable (cf. fig.1 and fig.2, second panel). Therefore, this experiment suggests the following reaction sequence:

mfVSG → 1,2-dimyristin →→ glycerol + 2 myristic acid
↘
sVSG

3.2. *Estimation of mfVSG hydrolase activity during transformation of bloodstream forms to procyclic cells*

The activity of mfVSG hydrolase in lysates of established procyclic cells of the variant clone MiTat 1.4 is less than 0.1% of that in lysates of bloodstream forms. Therefore, in procyclic cells the gene responsible for the synthesis of the enzyme is not expressed. The experiment detailed in fig.3 demonstrates that repression of enzyme synthesis occurs rapidly during transformation.

Bloodstream forms are grown overnight at 37°C in the presence of *cis*-aconitate + citrate (CCA). The cultures are then transferred to 27°C (cf. section 2.4). Under these conditions the cells rapidly repress VSG synthesis, release their surface coat into the medium, and divide as morphologically transformed procyclic cells [8,10]. The upper part of fig.3 shows the growth of the bloodstream forms at 37°C (●——● – 17 to 0 h) and the growth

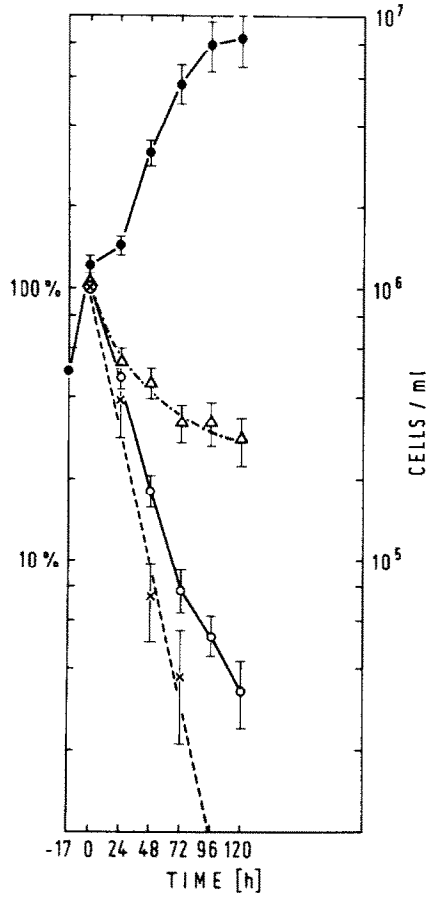

Fig.3. Estimation of mfVSG hydrolase during transformation. The upper part (●—●) shows the growth of the bloodstream forms at 37°C (−17 to 0 h) and of the transformed procyclic cells at 27°C (0–120 h). At various times samples were taken for the estimation of the percentage of coated cells by immunofluorescence (×---×). mfVSG hydrolase activity was assayed as described in the legend to fig.1. (○—○) Relative enzyme activity/cell (100% refers to a rate of hydrolysis of 1.35 nmol mfVSG·h^{-1}·10^{-8} cells); (△—△) relative enzyme activity/ml culture. Average values of 8 transformation experiments are given. Bars: SE.

of the transformed cells (●—●), 0 to 120 h). Coat release is monitored by the determination of the percentage of coated cells in the population (×—×); an exponential decrease with a half-time of about 14 h is observed. In the same time interval (0 to 120 h) enzyme activity rapidly declines. If expressed as a relative activity/cell, the decrease is

Table 1

Estimation of mfVSG hydrolysis in newly transformed procyclic cells

Time after temperature shift to 27°C (h)	Coated cells before/after protein A-Sepharose column (%)	Hydrolase activity before/after protein A-Sepharose column (%)
0	100	100
24	39 ± 11/2 ± 1	47.2 ± 5.2/39.4 ± 9.2
48	7.4 ± 2.4/<1%	18.0 ± 3.6/16.2 ± 3.8
72	3.8 ± 1.7/<0.5%	7.3 ± 2.4/4.8 ± 0.9

Culture samples were taken at various times after the temperature shift to 27°C (cf. fig.3). For the hydrolase activity 100% refers to a rate of hydrolysis of 1.35 nmol mfVSG·h^{-1}·10^{-8} cells

exponential ($t_{1/2} \approx 18$ h) during the first 72 h (○—○). If the same data are expressed as a relative rate/ml culture, enzyme activity decreases to 30% of the initial level in 120 h (△—△). Comparison of the curves for the percentage of coated cells in the culture (×—×) and for the relative enzyme content/cell (○—○) suggests that hydrolase activity is retained following loss of coat. To test this observation, coated and uncoated cells are separated in samples taken from cultures at 24, 48 and 72 h. After treatment with anti-sVSG antibody the cells are passed over a protein A affinity column which retains essentially all coated cells. The data in table 1 demonstrate that enzyme activity in the uncoated procyclic cells not retained by the column is very similar to that of the starting population. Thus, enzyme activity is retained in the procyclic cells even after shedding of their coat. Finally, no enzyme activity is detectable in the culture medium after removal of the transformed cells by centrifugation.

4. DISCUSSION

mfVSG hydrolase is an integral membrane-bound protein in bloodstream trypanosomes which converts mfVSG to sVSG + 1,2-dimyristoylglycerol. The membrane association of the enzyme has also been pointed out in independent studies by Turner [11] and by Ferguson and Cross [3,4]. As shown in this study the enzyme is inhibited by

$ClHgBzSO_3$. This observation is useful for the purification of native mfVSG.

The hydrolase is absent from procyclic cells in agreement with independent observations made in other laboratories ([11] and G.S. Lamont and G.A.M. Cross, personal communication). The experiments reported in fig.3 and table 1 are interpreted in the following way: upon transfer of the bloodstream forms to 27°C the synthesis of mfVSG hydrolase is rapidly repressed. Thus, cessation of the expression of the mfVSG hydrolase gene is part of the complex changes in gene activity occurring during differentiation of bloodstream forms to procyclic cells. Random enzyme partitioning during cell division is consistent with the observation that the procyclic cells retain enzyme activity through several divisions. However, the decline in enzyme activity, expressed as enzyme activity/cell (fig.3, ○—○) proceeds more rapidly than expected from the growth rate (●—●), assuming equal partitioning of the enzyme between daughter cells upon division. The decrease in enzyme activity, expressed as enzyme activity/ml culture (△—△), suggests that this may be a function of protein turnover. This suggestion is supported by the finding that no enzyme activity is lost, in an active form, during the release of surface coat.

Although the present results give no direct demonstration for the action of mfVSG hydrolase during coat release the enzyme remains a prime candidate for effecting this process because sVSG appears in the culture medium during shedding of the coat. Further information on the possible in vivo function of this enzyme may be expected from studies on its distribution in the cell before and during transformation.

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