Procyclin Gene Expression and Loss of the Variant Surface Glycoprotein during Differentiation of *Trypanosoma Brucei*

Isabel Roditi,‖ Heinz Schwarz,* Terry W. Pearson,‡ Robert P. Beecroft,‡ Margaret K. Liu,‡ Jennifer P. Richardson,‡ Hans-Jörg Bühring,§ Jürgen Pleiss,* Roland Bülow,* Richard O. Williams,‖ and Peter Overath*

*Max-Planck-Institut für Biologie, Tübingen, Federal Republic of Germany; ‡Department of Biochemistry and Microbiology, University of Victoria, Victoria, B.C., Canada; §Medizinische Klinik der Universität Tübingen, Tübingen, Federal Republic of Germany; and ‖Kernforschungzentrum Karlsruhe, Institut für Genetik und Toxikologie von Spaltstoffen, Karlsruhe, Federal Republic of Germany

Abstract. In the mammalian host, the unicellular flagellate *Trypanosoma brucei* is covered by a dense surface coat that consists of a single species of macromolecule, the membrane form of the variant surface glycoprotein (mfVSG). After uptake by the insect vector, the tsetse fly, bloodstream-form trypanosomes differentiate to procyclic forms in the fly midgut. Differentiation is characterized by the loss of the mfVSG coat and the acquisition of a new surface glycoprotein, procyclin. In this study, the change in surface glycoprotein composition during differentiation was investigated in vitro. After triggering differentiation, a rapid increase in procyclin-specific mRNA was observed. In contrast, there was a lag of several hours before procyclin could be detected. Procyclin was incorporated and uniformly distributed in the surface coat. The VSG coat was subsequently shed. For a single cell, it took 12–16 h to express a maximum level of procyclin at the surface while the loss of the VSG coat required ~4 h. The data are discussed in terms of the possible molecular arrangement of mfVSG and procyclin at the cell surface. Molecular modeling data suggest that a $(Asp\text{-}Pro)_2$ $(Glu\text{-}Pro)_{22\text{-}29}$ repeat in procyclin assumes a cylindrical shape 14–18 nm in length and 0.9 nm in diameter. This extended shape would enable procyclin to interdigitate between the mfVSG molecules during differentiation, exposing epitopes beyond the 12–15-nm-thick VSG coat.

Among the complex changes in gene expression and phenotype that accompany the differentiation of one cell type to another, alterations in the molecular composition of the cell surface are of particular interest because surface molecules form the basis for communication between cells and their environment. Parasitic protozoa that alternate in their life cycle between a mammalian host and an insect vector are subject to particularly dramatic changes in environment. It is therefore not unexpected that they respond with equally drastic changes in surface architecture. Indeed, African trypanosomes, exemplified here by *Trypanosoma brucei*, undergo extensive changes in surface composition. The kinetics of expression and replacement of two different surface molecules, characteristic of two developmental stages, are the subject of this communication.

In the mammal, bloodstream-form trypanosomes possess a 12–15-nm-thick coat that consists of ~10^7 molecules of the membrane form of the variant surface glycoprotein (mfVSG; Cross, 1975; Cardoso de Almeida and Turner, 1983; Ferguson et al., 1988) and that covers the entire parasite surface (Vickerman and Luckins, 1969). The mfVSG is derived from a polypeptide precursor that is the product of a single gene. Through the sequential expression of a large repertoire of VSG genes, coding for antigenically distinct proteins, the trypanosome is able to evade the host immune response (for reviews see Donelson, 1987; Van der Ploeg, 1987). The VSG serves a protective function by shielding underlying invariant membrane-embedded molecules.

Trypanosomes ingested by the tsetse fly as part of a blood meal transform to procyclic forms over a 2–3-d period (Vickerman, 1985). Transformation in vitro occurs with similar kinetics to transformation in vivo, giving rise to procyclic culture forms which, by several criteria, closely resemble those found in the insect midgut (Brown et al., 1973; Honigberg et al., 1976; Richardson et al., 1986; Pearson et al., 1987). During the transition from bloodstream to procyclic forms, VSG-specific mRNA and protein synthesis are repressed and the VSG is shed in a processed form into the medium (Overath et al., 1983; Ehlers et al., 1987; Bülow et

1. *Abbreviations used in this paper*: FACS, fluorescence-activated cell sorter; mfVSG, membrane form of the variant surface glycoprotein; VSG, variant surface glycoprotein.

al., 1988). The arising procyclic forms expose common antigens on the surface membrane (Seed, 1964; Barry and Vickerman, 1979). The only one of these antigens to have been characterized in any detail is an unusual glycoprotein that consists of a glutamic acid–proline dipeptide repeat for almost half its length (Roditi et al., 1987; Mowatt and Clayton, 1987; Richardson et al., 1988). This glycoprotein has recently been purified and named procyclin (Richardson et al., 1988). *T. brucei* contains a family of related procyclin genes, several of which are expressed (Roditi et al., 1987; Mowatt and Clayton, 1987; Mowatt and Clayton, 1988). These genes are also referred to as procyclic acidic repetitive protein genes by Mowatt and Clayton (1987, 1988). In contrast to the VSG in bloodstream forms, procyclin is antigenically conserved in all isolates of *T. brucei* tested (Richardson et al., 1986; Roditi et al., 1989). The function of procyclin still remains to be elucidated.

We have studied procyclin gene expression and the loss of VSG during differentiation (also known as transformation) in vitro. It is concluded that procyclin is synthesized and incorporated into the surface membrane before the VSG coat is lost.

Materials and Methods

Trypanosome Strains and Transformation Protocol

Trypanosome variant MITat 1.4, cloned from *T. brucei* stock 427 (Cross, 1975), was used throughout this study. The conditions used for the culture and transformation of trypanosomes in vitro are those described by Overath et al. (1986). In brief, bloodstream-form trypanosomes isolated from the blood of infected mice were grown at 37°C in trypanosome medium + 15% heat-inactivated FBS from an initial density of 4×10^5/ml to a density of 1.2–1.4 $\times 10^6$/ml above a feeder layer of Microtus embryo fibroblasts. At time 0 h differentiation was initiated by the addition of citrate/*cis*-aconitate and a reduction in temperature to 27°C. Within 24 h the parasite number increased to 2.6–3.7 $\times 10^6$/ml; within 48 h a density of 4–5 $\times 10^6$/ml was reached.

RNA Isolation and Northern Blots

Total RNA was isolated from $\sim 10^8$ trypanosomes at each timepoint after the start of transformation. Trypanosomes were centrifuged at 800 *g* for 15 min, the supernatant was discarded, and the trypanosomes were resuspended in ~100 μl medium that remained in the centrifuge tube. This suspension was added to a monophasic solution consisting of 500 μl 100 mM NaCl, 25 mM Tris-Cl, pH 7.4, 5 mM EDTA (NTE buffer) containing 1% SDS and 500 μl NTE-saturated phenol at 80°C and mixed gently for 3 min. After centrifugation for 10 min at 12,000 *g* in a microcentrifuge (Eppendorf centrifuges made by Brinkmann Instruments, Inc., Westbury, NY), the aqueous phase was recovered and extracted with an equal volume of phenol saturated with NTE. The mixture was centrifuged for 5 min, and the aqueous phase collected and extracted with an equal volume of chloroform. Nucleic acid in the aqueous phase was recovered by ethanol precipitation followed by centrifugation at 12,000 *g* for 15 min. RNA isolated in this way was free of detectable DNA.

Northern blot analysis was performed according to standard procedures. Total RNA was treated with glyoxal and DMSO (McMaster and Carmichael, 1977) and separated on a 1.3% agarose gel followed by transfer to nitrocellulose. The following plasmids were used as hybridization probes: pPRO2001, a procyclin cDNA clone (Roditi et al., 1987), and pBS211, a cDNA clone of a gene expressed at equal levels in bloodstream- and procyclic-form trypanosomes. Probes were labeled with ^{32}P by nick translation using standard procedures (Rigby et al., 1977).

Hybridization was quantitated by scanning densitometry of autoradiographs using an isodensitracer (model 3CS; Joyce Loebl, Gateshead, England) and by liquid scintillation counting (Kontron Elektronik GmbH, Zurich) of nitrocellulose strips. For each timepoint, the value obtained with pPRO2001 was normalized against that obtained with pBS211 and expressed as a percentage of the measurement obtained for established procyclic forms.

Antisera and mAbs

Anti-VSG Antibodies. mAT127 is a mAb that recognizes an epitope of VSG MITat 1.4 on the surface of live bloodstream form trypanosomes. It was isolated from the culture supernatant of the respective hybridoma line by protein A–Sepharose affinity chromatography. Monoclonal antibody mAT127 was used for the immunofluorescence and flow cytometry experiments. Immunoelectron microscopy was performed with a polyclonal rabbit antiserum against the soluble form of VSG MITat 1.4.

Antiprocyclin Antibodies. 10 mAbs of the IgG isotype, which recognize several surface epitopes of procyclin, have previously been described (Richardson et al., 1986; Liu and Pearson, 1987; Richardson et al., 1988). A cocktail containing all 10 mAbs was purified from pooled ascites fluid by protein A–Sepharose affinity chromatography and used for either immunofluorescence, flow cytometry, or immunoelectron microscopy.

PAGE

SDS-PAGE was performed under reducing conditions according to Laemmli (1970) using a mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA). Proteins were stacked using a 3% gel and separated on a 10% gel at 10 mA. Prestained molecular weight markers (catalog No. 6041LA; Bethesda Research Laboratories, Gaithersburg, MD) were run on each gel so that they could be seen on blotting membranes after electrophoretic transfer.

Transfer of proteins from SDS-PAGE gels onto microporous membranes and subsequent detection of antigens were performed by the procedure of Towbin et al. (1979) extensively modified to reduce background binding of antibodies (Birk and Koepsell, 1987), to allow blotted proteins to renature (Bestagno et al., 1987), and to increase sensitivity by adding an additional antibody as a sandwich reagent. This method is described in detail elsewhere (Richardson et al., 1988), except that the membrane used was Immobilon (Millipore Continental Water Systems, Bedford, MA) and the ascites fluids containing the control mAb (α-urokinase) or the antiprocyclin mAb TBRPI/247 (Richardson et al., 1988) were used on the blots at a dilution of 1:500 for 18 h at 4°C. In addition, a second antibody was included after the mAb TBRPI/247 and the antiurokinase mAb. This was a 1:5,000 dilution of rabbit anti-mouse IgG serum for 3 h at room temperature. Detection of this second antibody was performed by incubating the washed blots with 5.1×10^7 cpm of ^{125}I-labeled horse anti-rabbit IgG $F(ab)_2$. After washing and drying, the Immobilon sheets were exposed for autoradiography. Autoradiographs were developed after 9 d, cut into strips and scanned at 540 nm in a scanning spectrophotometer (DU; Beckman Instruments, Inc., Palo Alto, CA) fitted with a chart recorder (model 6051; Gilford, Oberlin, OH).

Determination of mfVSG

Aliquots of trypanosomes in culture were chilled on ice, centrifuged, resuspended in 1 ml cold trypanosome medium, and centrifuged again. After resuspension in 1 ml trypanosome medium, trypanosomes were counted, the sample was centrifuged again, and the pellet was stored at −20°C. Equal numbers of trypanosomes were processed for SDS-PAGE. After staining, proteins were quantified in a transmission densitometer (Pharmacia-LKB, Freiburg, FRG). The height of the VSG peak was corrected for colocalizing proteins by subtracting the absorbance given by a lysate of procyclic forms.

Immunofluorescence and Two-Color Flow Cytometry

Fluorophore labeling of antibodies was performed as described by Goding (1983). Monoclonal antibody mAT127 (2.8 mg IgG) was labeled with 250 μg FITC in 3.5 ml sodium carbonate buffer, pH 9.5, for 2 h at room temperature. A mixture of 10 antiprocyclin mAbs (4.8 mg IgG) was labeled with 360 μg TRITC in 2 ml of this buffer for 2 h at room temperature. Labeled antibodies were separated from free fluorophore by gel exclusion chromatography on Sephadex G25 (Pharmacia-LKB).

Fluorescence microscopy and flow cytometry were performed on fixed trypanosomes. 10^7 trypanosomes were pelleted by centrifugation and resuspended in 0.5 ml PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2). After addition of 0.5 ml 4% formaldehyde, 0.1% glutaraldehyde, trypanosomes were stored for 12 h at 5°C. Trypano-

somes were washed in PBS/2% BSA, resuspended in this buffer, incubated for 1–2 h at 0°C with FITC-mAT127 and TRITC-antiprocyclin mAbs, and, finally, washed twice.

The fluorescence of trypanosomes was examined in a Leitz Ortholux microscope using an NPL FLUOTAR objective. For flow cytometry, trypanosomes were analyzed on a fluorescence-activated cell sorter (FACS) IV (Becton Dickinson & Co., Sunnyvale, CA). The green fluorescence of FITC was excited in a 5-W argon laser at 488 nm and emitted fluorescence was detected through a 525-nm band pass filter by an EMI 9524 photomultiplier. The red fluorescence of TRITC was excited with a 0.8-W krypton laser at 568 nm and emitted fluorescence was detected through a 590-nm-long pass filter and a 610-nm band pass filter by a EMI 9798 photomultiplier.

Immunoelectron Microscopy

Preembedding labeling. 10^7 trypanosomes were centrifuged and resuspended in 0.5 ml trypanosome medium + heat-inactivated FBS at 27°C. After addition of 50 μg antiprocyclin mAbs, the trypanosomes were incubated for 30 min at 27°C. After two washes with trypanosomes medium + heat-inactivated FBS at room temperature, the trypanosomes were resuspended in 0.5 ml PBS and fixed by addition of 0.5 ml 4% formaldehyde. The bound antibodies were detected with protein A–5-nm gold complexes prepared as described (Mühlpfordt, 1982; Slot and Geuze, 1985 *a*,*b*). Subsequently, the trypanosomes were fixed with 2.5% glutaraldehyde, 1% tannic acid in 0.1 M cacodylate, pH 6.9 (Simionescu and Simionescu, 1976), for 2 h at 4°C, embedded in agarose, postfixed with 1% osmium tetroxide in 0.1 M cacodylate, dehydrated with ethanol, and finally embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in an electron microscope (model 201; Philips Electronic Instruments, Inc., Mahwah, NJ).

On-Section Labeling. Trypanosomes were fixed in 2% formaldehyde, 0.05% glutaraldehyde as described for immunofluorescence. After embedding the trypanosomes in agarose, the samples were dehydrated with ethanol at progressively lower temperature and embedded in Lowicryl HM20 at −35°C (Carlemalm et al., 1982). Ultrathin sections were treated with 0.5% BSA, 0.2% gelatin in PBS to block nonspecific binding sites, incubated with antiprocyclin mAbs or rabbit anti-VSG antiserum and protein A–15-nm gold complexes. The labeled sections were stained and screened as described above.

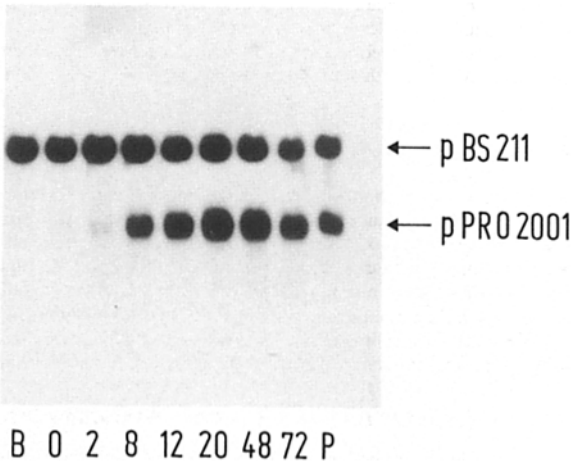


Figure 1. Northern blot analysis of procyclin mRNA expression during trypanosome differentiation. Total RNA isolated from bloodstream-form trypanosomes (*B*), procyclic forms (*P*), and from trypanosomes 0 to 72 h after the start of transformation was separated on a 1.3% agarose gel and transferred to nitrocellulose. The blot was probed with a mixture of ^{32}P-labeled plasmids pPRO2001 and pBS211. In the autoradiograph, pBS211 detects a transcript expressed in equal amounts in bloodstream- and in procyclic-form trypanosomes, and serves as a reference for quantitation of the procyclin mRNA detected by pPRO2001.

Molecular Dynamics Calculation of Protein Structure

Molecular dynamics calculations were carried out using the GROMOS program (Van Gunsteren and Karplus, 1982; Van Gunsteren et al., 1983) with the bond lengths constrained by the SHAKE algorithm (Ryckaert et al., 1977) and a cut-off for nonbonded interactions at 1 nm. After energy minimization of the initial conformations, molecular dynamics simulations (time step 2 fs) were performed with coupling to a heat bath at 300 K (Berendsen et al., 1984). Two time constants, 10 and 100 fs, were used to simulate tight and loose coupling, respectively, because tight coupling increases the transition rate between two conformations whereas loose coupling allows a realistic description of the equilibrium dynamics (Van Gunsteren et al., 1983; Berendsen et al., 1984). The simulations were performed on a Microvax II and the graphics on a PS390 with the graphics program SYBYL/MENDYL (Evans & Sutherland, Salt Lake City, UT).

Results

Expression of Procyclin and Loss of VSG during Transformation

To investigate the kinetics of induction of procyclin gene expression, RNA was isolated from trypanosomes at various times after triggering differentiation, and also from bloodstream forms and established procyclic culture forms of the same variant. Northern blot analysis (Fig. 1) was performed using a full-length cDNA clone of procyclin (pPRO2001) as a probe. A second cDNA clone (pBS211) that is not differentially expressed in bloodstream- and procyclic-form trypanosomes was included as a control probe. Although there was some variation between individual experiments, procyclin transcripts were clearly visible 2 h after the start of transformation. The relative amount of procyclin mRNA initially increased linearly with time, reaching the level observed in established procyclic forms within 18–24 h (Fig. 2). RNA isolated from bloodstream forms, either directly or after growth in culture overnight at 37°C, showed little or no detectable procyclin mRNA.

The total amount of procyclin present in trypanosomes at various times after the onset of transformation was assayed by immunoblot analysis using a mAb that recognizes the dipeptide repeat of procyclin (Richardson et al., 1988). In contrast to the mRNA, which can be detected within 2 h, the

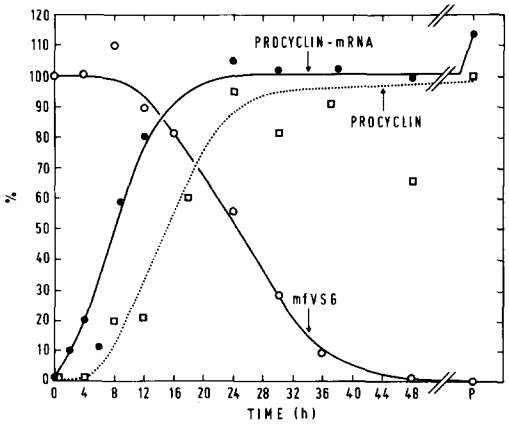


Figure 2. Changes in the levels of procyclin mRNA, procyclin, and mfVSG during differentiation. Quantitation of the samples was performed as described in Materials and Methods.

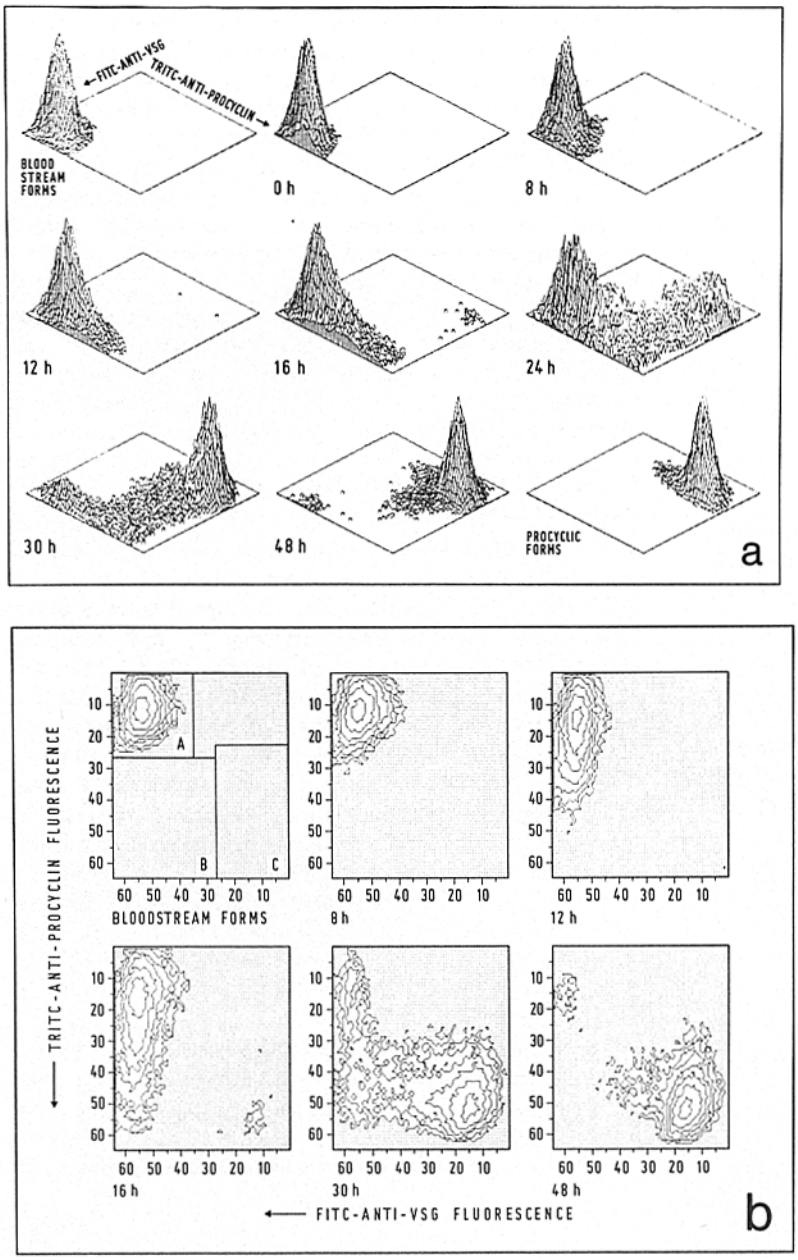


Figure 3. (*a*) Analysis by two-color flow cytometry of surface VSG and procyclin during differentiation of trypanosomes. The logarithm of the fluorescence intensity for FITC-anti-VSG and TRITC-anti-procyclin is plotted as indicated on the panel for bloodstream forms. The third dimension refers to the cell number. For every sample 50,000 cells were analyzed. For clarity, a small and variable fraction ($\leqslant 0.5 \leqslant 2\%$) of cells not labeled by either fluorochrome was eliminated from the panels. This area could also contain debris from the feeder cells. (*b*) Two-dimensional diagrams of representative panels shown above. The data are displayed on a 64–64 channel matrix. Contour lines were set to represent 4, 8, 16, 32, 64, . . . cells, respectively. Areas designated A to C in the bloodstream-form panel refer to populations of $VSG^+procyclin^-$, $VSG^+procyclin^+$, and $VSG^-procyclin^+$ trypanosomes, respectively.

procyclin protein was first detectable after 8 h, reaching maximum levels within 24 h (Fig. 2).

The amount of cell-associated mfVSG at different times during transformation was estimated by SDS-PAGE. Membrane-form VSG was slowly lost during the initial 12–16 h after the start of transformation and then became undetectable within the next 32–36 h (Fig. 2). Comparison of the timecourse of procyclin synthesis and VSG loss indicated that the rise in the level of procyclin occurred when most of the VSG was still cell associated.

Surface Expression of Procyclin and VSG Analyzed by Two-Color Flow Cytometry

So far, the above biochemical and immunochemical analysis provides information on the average level of procyclin and VSG in the trypanosome population as a function of time. The kinetics shown in Fig. 2 depend on two parameters: (a) the rate at which a trypanosome can synthesize procyclin and release VSG; and (b) the fraction of the population contributing to the overall change in antigen composition at any given time. These parameters can be separated by studying the distribution of the two surface glycoproteins by flow cytometry. Trypanosomes were labeled simultaneously with an FITC-conjugated anti-VSG mAb and a TRITC-conjugated antiprocyclin mAb mixture and analyzed by FACS. The two-dimensional FACS analysis is depicted in Fig. 3 and quantitated in Fig. 4. Initially, the trypanosomes were only labeled with the anti-VSG mAb (Fig. 3, panels for bloodstream forms and 0 h). During the subsequent 8 h after the initiation of transformation, this situation was unchanged. By 12 h, ~8% of the population had acquired procyclin at the surface (Figs.

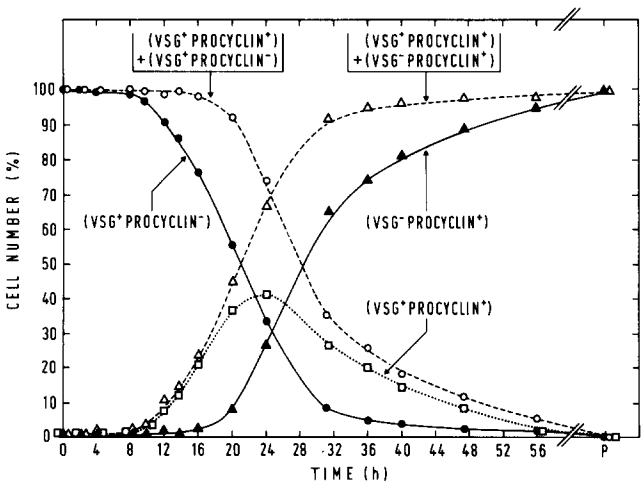


Figure 4. Percentage of VSG⁺procyclin⁻, VSG⁺procyclin⁺, and VSG⁻procyclin⁺ trypanosomes in the population during differentiation. Integral cells counts were plotted as a function of time. The data were compiled from the flow cytometry experiment shown in Fig. 3 and from two other experiments.

3 and 4) without a detectable change in the labeling intensity of the VSG coat. By 16 h, ~20% of the trypanosomes became positive for both antigens; some of these showed a TRITC-fluorescence intensity comparable to that of established procyclic forms. Also, between 12 and 16 h, a significant number of trypanosomes devoid of VSG appeared. The remaining panels demonstrate the further progression of the population from a VSG⁺procyclin⁻ state via an intermediate VSG⁺procyclin⁺ state to the VSG⁻procyclin⁺ state. Finally, a small fraction of the population retained the VSG⁺procyclin⁻ phenotype and the morphology of bloodstream forms when most trypanosomes had transformed (Fig. 3, 48 h). By integrating the three cell populations for each sample the curves depicted in Fig. 4 were obtained.

It is clear from Figs. 3 and 4 that the trypanosomes do not transform in synchrony. While some trypanosomes are readily transformed, others lag behind by 24–30 h and some do not transform at all. A fraction of the trypanosomes expressed procyclin at the surface 8–12 h after the start of differentiation in agreement with the time procyclin first became detectable by immunoblotting (Fig. 2). After 16 h, these trypanosomes showed a TRITC-fluorescence intensity comparable to that of mature procyclic forms, suggesting that it took 12–16 h for a single organism to express a high level of procyclin at the surface. The fraction of VSG⁺procyclin⁺ intermediates reached a maximum of 40% of the population at 24 h. The observation that a significant fraction of VSG⁻procyclin⁺ trypanosomes appeared between 12 and 16 h suggests that a trypanosome can release its VSG coat in a time as short as 4 h. There is also good agreement between the time required to release half the total VSG in the population (Fig. 2, 25 h) and the decrease in the number of VSG⁺ trypano-

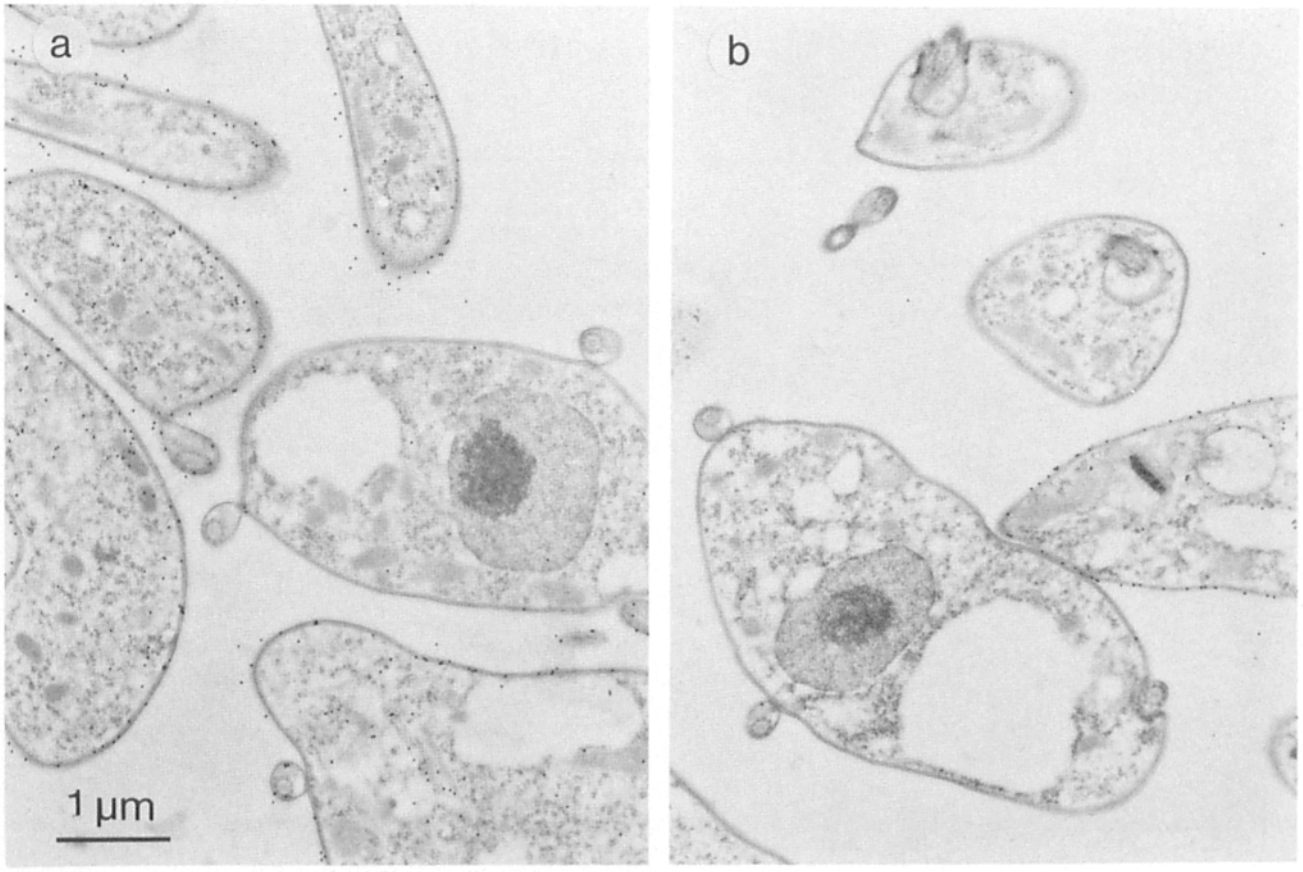


Figure 5. Electron micrograph of on-section labeling of Lowicryl-embedded trypanosomes 12 h after triggering transformation. Rabbit anti-VSG polyclonal antibodies (*a*) or murine antiprocyclin mAbs (*b*) were detected with protein A–15-nm gold particles.

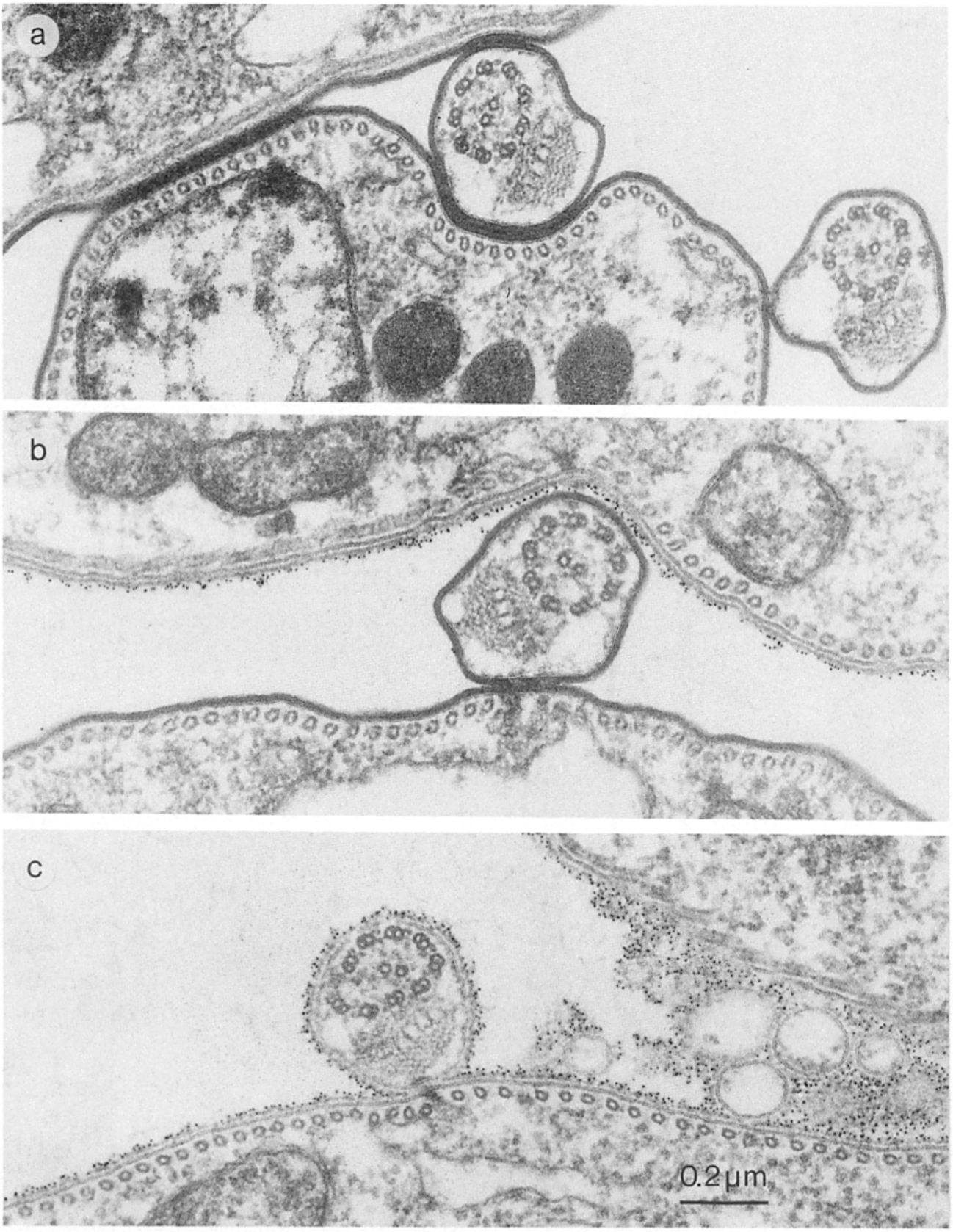
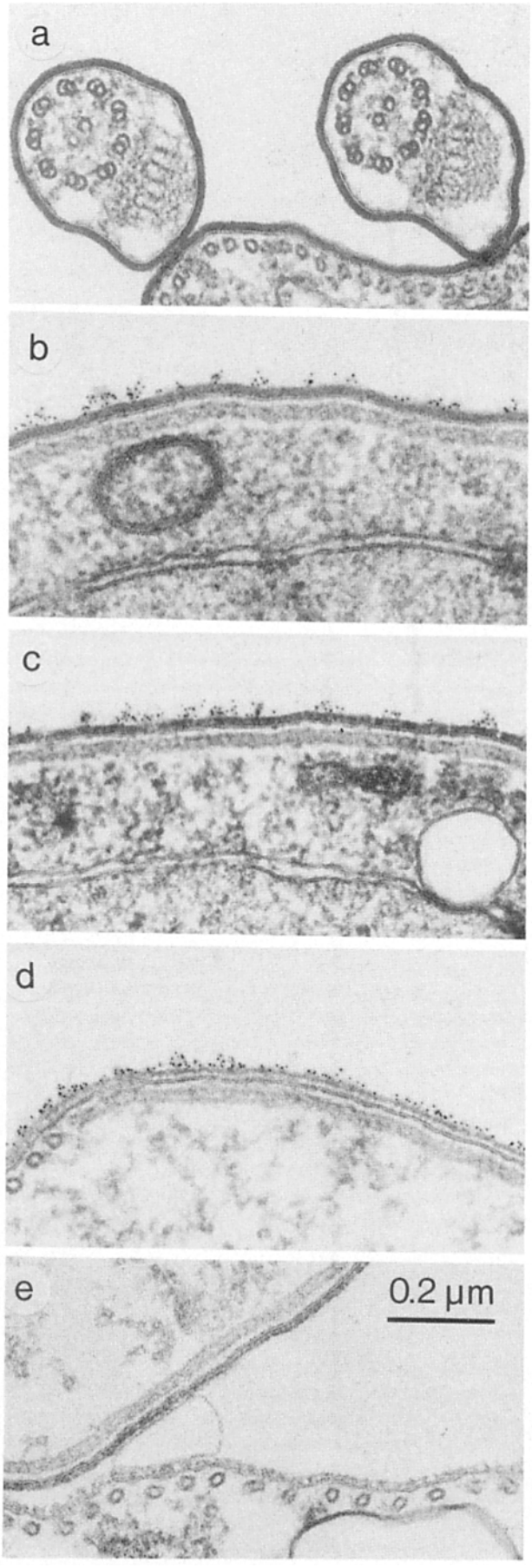


Figure 6. Electron micrograph of trypanosomes labeled with antiprocyclin mAbs and protein A–5-nm gold before embedding. Trypanosomes were sampled 0 (*a*), 12 (*b*), and 48 h (*c*) after triggering transformation. The surface coat was stained by tannic acid.



somes to half the initial value (25.7 ± 2.5 h by FACS and ~26 h by fluorescence microscopy). These results indicate that the appearance of procyclin on the cell surface precedes the loss of the VSG coat.

Localization of Procyclin and VSG by Electron Microscopy

The synthesis of procyclin and the release of VSG in differentiating trypanosomes were studied at the ultrastructural level by immunoelectron microscopy. Trypanosomes harvested 12 h after triggering transformation were embedded in Lowicryl HM20 and the sections labeled with anti-VSG antibodies (Fig. 5 *a*) or antiprocyclin mAbs (Fig. 5 *b*) and protein A-gold. As expected from the flow cytometric experiments (Figs. 3 and 4), the majority of trypanosomes at 12 h could be labeled with anti-VSG antibodies, mainly at the surface (Fig. 5 *a*), and only the occasional trypanosome remained devoid of label. In contrast, a few trypanosomes labeled with antiprocyclin mAbs while all others did not bind these antibodies (Fig. 5 *b*). In common with the VSG, procyclin was mainly located and uniformly distributed in the plasma membrane; no sections were observed that were labeled only in intracellular structures and not at the plasma membrane. The uniform distribution of procyclin over the entire body of the trypanosomes was also evident from microscopic inspection of double-fluorescent trypanosomes labeled simultaneously with TRITC-antiprocyclin and FITC-anti-VSG mAbs (data not shown).

The coexistence of VSG and procyclin at the cell surface could be demonstrated by a preembedding labeling experiment. Trypanosomes were first labeled with antiprocyclin mAbs in medium at 27°C. After washing and fixation, bound antibodies were tagged with protein A-gold and the surface coat was stained with tannic acid. Representative sections of trypanosomes obtained by this labeling protocol are shown in Fig. 6. Bloodstream forms (Fig. 6 *a*) were surrounded by a distinct coat, a uniformly stained, 12–15 nm-thick layer adjacent to the plasma membrane. After 48 h (Fig. 6 *c*), the surface architecture had changed considerably. Tannic acid staining revealed a light halo, 8–10 nm wide, separating a layer of moderately densely staining material from the membrane. This outer layer was heavily labeled with protein A-gold. The micrograph of Fig. 6 *b* allows direct comparison of these two forms. In Fig. 7, these two forms (*a* and *d*) are compared to two intermediates. Significantly, the 12-h sample contained a considerable proportion of trypanosomes that labeled with protein A-gold and at the same time showed an apparently intact VSG coat (Fig. 7 *b*). In addition, procyclin$^+$ trypanosomes could be detected in which the VSG coat had a fuzzy or gapped appearance (Fig. 7 *c*) suggesting that these cells were in the process of VSG release.

Figure 7. Electron micrograph of trypanosomes labeled 12 h after triggering transformation. Preembedding labeling with antiprocyclin mAbs and protein A–5-nm gold and staining of the surface coat with tannic acid were performed as for Fig. 6. Sections were obtained showing an intact VSG coat and no procyclin (*a*), both VSG and procyclin (*b* and *c*) or procyclin, but no detectable VSG coat (*d*). In part *e*, mAbs were omitted.

Finally, a control preparation in which the trypanosomes were not incubated with antiprocyclin antibodies was completely devoid of protein A–gold particles (Fig. 7 *e*).

Molecular Modeling of the Dipeptide Repeat of Procyclin

The structure of the glutamic acid–proline repeat in procyclin was investigated by molecular dynamics techniques. Simulations were performed for 400 ps with the model peptide Pro_1-Glu-Pro-Glu-Pro_5. Two different initial conformations with all *trans* peptide bonds, α-helical ($\varphi = -57°$, $\psi = -47°$; rise per residue, 0.15 nm) or extended ($\varphi = \psi = 180°$; rise per residue, 0.36 nm), were assumed, neither of them containing net charges. The extended structure quickly contracted to a structure with a rise per residue of 0.29 nm with the central dihedral angles $\varphi_{Pro3} = -42°$, $\psi_{Pro3} = 118°$, $\varphi_{Glu} = -103°$, $\psi_{Glu} = 123°$, values averaged over 40 ps. This structure was stable under all coupling conditions. The α-helical conformation simulated under loose coupling conditions stretched to a rise per residue of 0.25 nm and eventually converged to the same structure as the extended conformation under tight coupling. Thus, an identical structure with a rise per residue of 0.29 nm was obtained starting from either the extended or α-helical conformation. An $(Asp\text{-}Pro)_2(Glu\text{-}Pro)_{29}$ repeat was modeled using the dihedral angles of the peptide given above. The resulting structure is a cylinder 18 nm in length and 0.9 nm in diameter (Fig. 8).

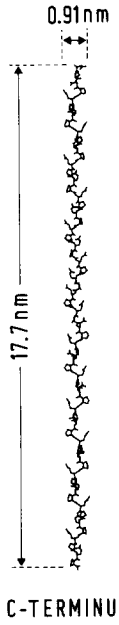


Figure 8. Structure of an $(Asp\text{-}Pro)_2$ $(Glu\text{-}Pro)_{29}$ peptide, derived by molecular dynamics simulation.

Discussion

Kinetics of Procyclin Synthesis and VSG Coat Release

Differentiation from bloodstream forms to procyclic forms in vitro is initiated by subjecting a culture of exponentially growing bloodstream forms to a decrease in temperature in the presence of citric acid cycle intermediates. The data from the FACS analysis clearly shows that transformation is not synchronous in these cultures. One possibility is that trypanosomes are only capable of responding to differentiation signals at a certain phase of the cell cycle and that a fraction of the population might take a considerable time to reach this phase. If this were the case, the observed lack of synchrony would be largely caused by variability in the timepoint of initiation rather than variability in the time required for completing transformation after triggering.

The results presented here show that procyclin mRNA synthesis is rapidly induced in trypanosomes differentiating from bloodstream to procyclic forms and precedes any obvious morphological changes. Interestingly, there is a lag of several hours between the detection of procyclin mRNA and detection of the protein by immunoblotting, FACS analysis, or immunoelectron microscopy, suggesting posttranscriptional control. It is possible that an additional signal is required in order for translation of procyclin mRNA to occur. Posttranscriptional control has also been observed in other developmentally regulated genes in lower eukaryotes including the tubulin genes of *Leishmania mexicana* (Wallach et al., 1982) and the cysteine proteinase 2 gene of *Dictyostelium discoideum* (Pears and Williams, 1987).

FACS analysis shows that an individual trypanosome takes at least 12 h to express maximum surface levels of procyclin (Fig. 3 *b*). As judged by fluorescence intensity, this can occur in the presence of the full complement of VSG. Immunoelectron microscopy (Fig. 7) also shows that procyclin can be found on trypanosomes where tannic acid staining reveals an essentially intact VSG coat. A lag in the onset of VSG release might be beneficial for survival. It has previously been demonstrated that bloodstream forms are resistant to lysis by host serum components whereas procyclic forms or bloodstream forms whose VSG coat has been digested with trypsin are susceptible to complement-mediated lysis by the alternative pathway (for review see Vickerman, 1985). If complement were to remain active for any length of time in the tsetse fly midgut, it could be detrimental for trypanosomes to shed the VSG coat too rapidly. If, in addition, procyclin is necessary for survival in the fly, parasites simultaneously expressing both VSG and procyclin on the surface could be at a selective advantage during differentiation.

It has recently been shown that VSG release mainly leads to its appearance as an NH_2-terminal fragment in the culture supernatant (Bülow et al., 1989). The time at which this fragment is detected correlates with the first appearance of VSG^- trypanosomes as determined by FACS analysis. That VSG release can occur in as little as 4 h for an individual trypanosome implies an active process with the NH_2-terminal fragment as the end-product.

Structure of Procyclin and Its Arrangement in the VSG Coat

Procyclin is expressed in at least two closely related versions (Roditi et al., 1987; Mowatt and Clayton, 1987; Richardson et al., 1988; Mowatt and Clayton, 1988). These consist of a 31-amino acid NH_2-terminal domain, containing a glycosylation site, followed by the sequence $(Asp\text{-}Pro)_2(Glu\text{-}Pro)_{22\text{-}29}$ and a 23-amino acid hydophobic tail. At present it is not known how procyclin is attached to the cell membrane. While the COOH-terminal sequence is a candidate for glypiation

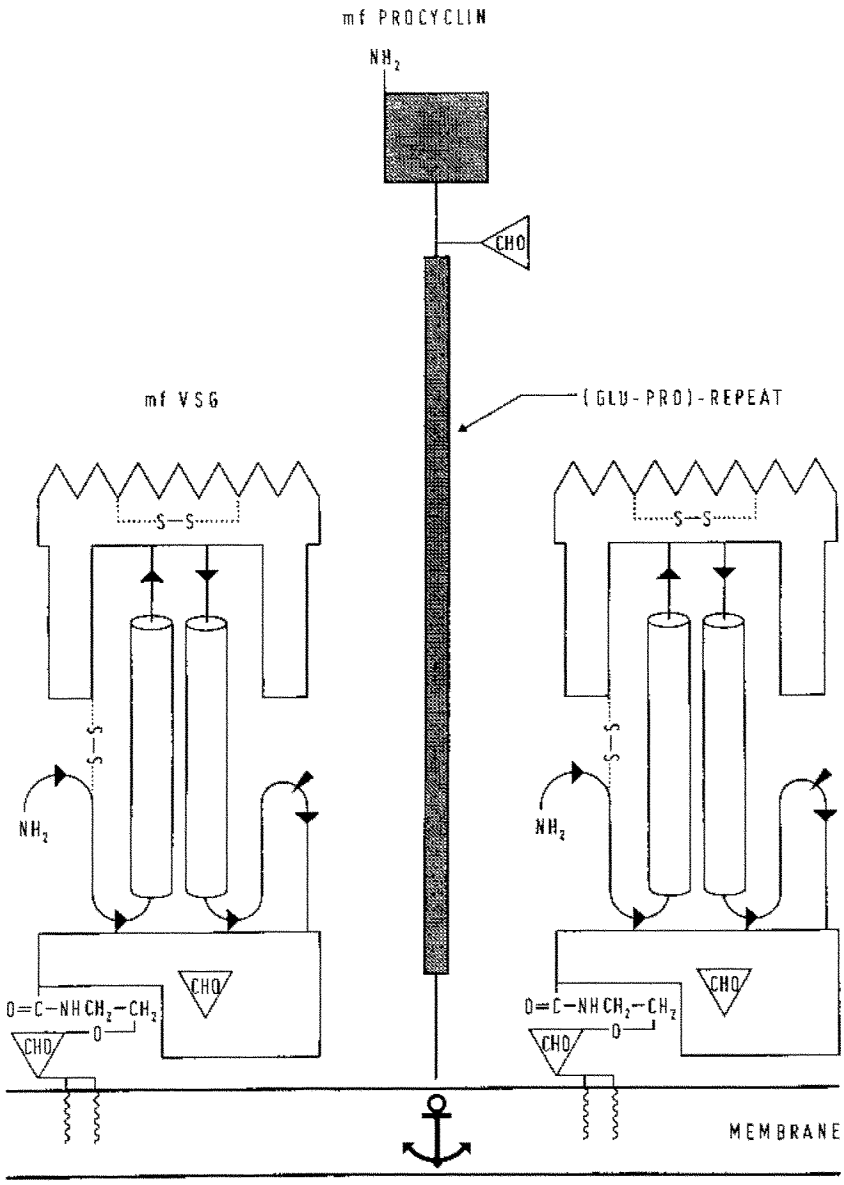


Figure 9. Model for coexpression of VSG and procyclin on the surface of differentiating trypanosomes. The model for mfVSG is taken from Jähnig et al. (1987). The membrane anchor of procyclin is represented schematically as its structure is unknown.

(Ferguson and Williams, 1988), it is also possible that it forms an α-helix which anchors the protein in the membrane.

It has previously been determined by nuclear magnetic resonance that glutamic acid–proline dipeptide repeats have an extended rodlike structure (Evans et al., 1986). We have used molecular modeling techniques to predict the dimensions of the dipeptide repeat in procyclin. Interestingly, the two different initial conformations that were assumed, α-helical and extended, converged to a single structure with a rise per residue of 0.29 nm. The resulting structure of the peptide $(Asp\text{-}Pro)_2(Glu\text{-}Pro)_{22\text{–}29}$ is a cylinder 14–18 nm in length and 0.9 nm in diameter. This finding, which is in good agreement with the estimates of interproton distances and dihedral angles measured by Evans et al. (1986), suggests that the repetitive region confers a highly extended structure upon procyclin. It is known that VSG molecules have an elongated structure dominated by two 8-nm-long helices in the NH_2-terminal domain (Freymann et al., 1984; Metcalf et al., 1987) that is connected via the COOH-terminal domain to the glycosyl-phosphatidylinositol membrane anchor (Ferguson et al., 1985; Schmitz et al., 1987; Ferguson et al., 1988). The length of the NH_2-terminal domain is ~10 nm, which is consistent with the 12–15-nm-thick coat observed by electron microscopy (Vickerman and Luckins, 1969). On the basis of these two structures, a hypothetical arrangement of mfVSG and procyclin on the surface of differentiating trypanosomes is proposed (Fig. 9) in which the procyclin repeat region interdigitates between and extends beyond the VSG molecules.

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References

Barry, J. D., and K. Vickerman. 1979. *Trypanosoma brucei*: loss of variable antigens during transformation from bloodstream to procyclic forms. *Exp. Parasitol.* 48:313–324.

Berendsen, H. J. C., J. P. M. Postma, W. F. van Gunsteren, A. DiNola, and J. R. Haak. 1984. Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* 81:3684–3690.

Bestagno, M., A. Cerina, S. Riva, and G. C. B. A. Ricotti. 1987. Improvement of Western blotting to detect monoclonal antibodies. *Biochem. Biophys. Res. Commun.* 146:1509–1514.

Birk, H. W., and H. Koepsell. 1987. Reaction of monoclonal antibodies with plasma membrane proteins after binding on nitrocellulose: renaturation of antigenic sites and reduction of non-specific antibody binding. *Anal. Biochem.* 164:12–22.

Brown, R. C., D. A. Evans, and K. Vickerman. 1973. Changes in oxidative metabolism and ultrastructure accompanying the differentiation if the mitochondrion in *Trypanosoma brucei*. *Int. J. Parasitol.* 3:691–704.

Bülow, R., C. Nonnengässer, and P. Overath. 1989. Release of the variant surface glycoprotein during differentiation of bloodstream to procyclic forms of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 32:85–92.

Cardoso de Almeida, M. L., and M. J. Turner. 1983. The membrane form of variant surface glycoproteins of *Trypanosoma brucei*. *Nature (Lond.)*. 302: 349–352.

Carlemalm, E., R. M. Garavito, and W. Villiger. 1982. Resin development for electron microscopy and analysis of embedding at low temperature. *J. Microsc. (Oxf.)*. 126:123–143.

Cross, G. A. M. 1975. Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. *Parasitology*. 71:393–417.

Donelson, J. E. 1987. Antigenic variation in African trypanosomes. *Contrib. Microbiol. Immunol.* 8:138–175.

Ehlers, B., J. Czichos, and P. Overath. 1987. RNA turnover in *Trypanosoma brucei*. *Mol. Cell. Biol.* 7:1242–1249.

Evans, J. S., B. A. Levine, I. P. Trayer, C. J. Dorman, and C. F. Higgins. 1986. Sequence-imposed structural restraints in the Ton B protein of *E. coli*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 208:211–216.

Ferguson, M. A. J., and A. F. Williams. 1988. Cell surface anchoring of proteins via glycosyl-phosphatidylinositol residues. *Annu. Rev. Biochem.* 57: 285–320.

Ferguson, M. A. J., M. G. Low, and G. A. M. Cross. 1985. Glycosyl-sn-1,2-dimyristyl phosphoinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 260:14547–14555.

Ferguson, M. A. J., S. W. Homans, R. A. Dwek, and T. W. Rademacher. 1988. Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface antigen to the membrane. *Science (Wash. DC)*. 239: 753–759.

Freyman, D., P. Metcalf, M. J. Turner, and D. Wiley. 1984. 6Å resolution X-ray structure of a variable surface glycoprotein from *Trypanosoma brucei*. *Nature (Lond.)*. 311:167–169.

Goding, J. W. 1983. Monoclonal Antibodies: Principle and Practice. Academic Press Inc., Orlando, FL. 228 pp.

Honigberg, B. M., I. Cunningham, H. A. Stanley, K. E. Su-Lin, and A. G. Luckins. 1976. *Trypanosoma brucei*: antigenic analysis of bloodstream, vector and culture stages by the quantitative fluorescent antibody methods. *Exp. Parasitol.* 33:496–502.

Jähnig, F., R. Bülow, T. Baltz, and P. Overath. 1987. Secondary structure of the variant surface glycoproteins of trypanosomes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 221:37–42.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.

Liu, M. K, and T. W. Pearson. 1987. Detection of circulating trypanosomal antigens by double antibody ELISA using antibodies to procyclic trypanosomes. *Parasitology*. 95:277–290.

McMaster, G. K., and G. G. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA*. 74:4835–4838.

Metcalf, P., M. Blum, D. Freymann, M. Turner, and D. C. Wiley. 1987. Two variant surface glycoproteins of *Trypanosoma brucei* of different sequence classes have similar 6Å resolution X-ray structures. *Nature (Lond.)*. 325: 84–86.

Mowatt, M. R., and C. E. Clayton. 1987. Developmental regulation of a novel repetitive protein of *Trypanosoma brucei*. *Mol. Cell Biol.* 7:2833–2844.

Mowatt, M. R., and C. E. Clayton. 1988. Polymorphism in the procyclic acidic repetitive protein (PARP) gene family of *Trypanosoma brucei*. *Mol. Cell Biol.* 8:4055–4062.

Mühlpfordt, H. 1982. The preparation of colloidal gold particles using tannic acid as an additional reducing agent. *Experientia*. 38:1127–1128.

Overath, P., J. Czichos, U. Stock, and C. Nonnengässer. 1983. Repression of glycoprotein synthesis and release of surface coat during transformation of *Trypanosoma brucei*. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:1721–1728.

Overath, P., J. Czichos, and C. Haas. 1986. The effect of citrate/cis-aconitate on oxidative metabolism during transformation of *Trypanosoma brucei*. *Eur. J. Biochem.* 160:175–182.

Pears, C. J., and J. G. Williams. 1987. Identification of a DNA sequence element required for efficient expression of a developmentally regulated and cAMP-inducible gene of *Dictyostelium discoideum*. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:195–200.

Pearson, T. W., S. K. Moloo, and L. Jenni. 1987. Culture form and tsetse fly midgut form procyclic *Trypanosoma brucei* express common proteins. *Mol. Biochem. Parasitol.* 25:273–278.

Richardson, J. P., L. Jenni, R. P. Beecroft, and T. W. Pearson. 1986. Procyclic tsetse fly midgut forms and culture forms of African trypanosomes share stage- and species-specific surface antigens identified by monoclonal antibodies. *J. Immunol.* 136:2259–2264.

Richardson, J. P., R. P. Beecroft, D. L. Tolson, M. K. Liu, and T. W. Pearson. 1988. Procyclin: an unusual immunodominant glycoprotein surface antigen from the procyclic stage of African trypanosomes. *Mol. Biochem. Parasitol.* 31:203–216.

Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* using DNA polymerase I. *J. Mol. Biol.* 113:237–251.

Roditi, I., M. Carrington, and M. Turner. 1987. Expression of a polypeptide containing a dipeptide repeat is confined to the insect stage of *Trypanosoma brucei*. *Nature (Lond.)*. 325:272–274.

Roditi, I., D. Dobbelaere, R. O. Williams, W. Masterson, R. P. Beecroft, J. P. Richardson, and T. W. Pearson. 1989. Expression of *Trypanosoma brucei* procyclin as a fusion protein in *E. coli*. *Mol. Biochem. Parasitol.* In press.

Ryckaert, J. P., G. Ciccotti, and H. J. C. Berendsen. 1977. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. *J. Comp. Physiol.* 23:327–341.

Schmitz, B., R. A. Klein, I. A. Duncan, H. Egge, J. Gunawan, J. Peter-Katalinic, U. Dabrowski, and J. Dabrowski. 1987. MS and NMR analysis of the cross-reacting determinant glycan of *Trypanosoma brucei brucei* MITat 1.6 variant surface glycoprotein. *Biochem. Biophys. Res. Commun.* 146:1055–1063.

Seed, J. R. 1964. Antigenic similarity among culture forms of the "brucei" group of trypanosomes. *Parasitology*. 54:593–596.

Simionescu, N., and M. Simionescu. 1976. Galloylglucoses of low molecular weight as mordant in electron microscopy. *J. Cell Biol.* 70:608–621.

Slot, J. W., and H. J. Geuze. 1985*a*. A new method of preparing gold probes for multiple-labelling microscopy. *J. Cell Biol.* 70:608:621.

Slot, J. W., and H. J. Geuze. 1985*b*. A new method of preparing gold probes for multiple-labelling cytochemistry. *Eur. J. Cell Biol.* 38:87–93.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose: procedure and some applications. *Proc. Natl. Acad. Sci. USA*. 76:4350–4354.

Van der Ploeg, L. H. T. 1987. Control of variant surface glycoprotein switching in trypanosomes. *Cell*. 51:159–161.

Van Gunsteren, W. F., and M. Karplus. 1982. Effect of constraints on the dynamics of macromolecules. *Macromol Rev.* 15:1528–1544.

Van Gunsteren, W. F., H. J. C. Berendsen, J. Hermans, W. G. J. Hol, and J. P. M. Postma. 1983. Computer simulation of the dynamics of hydrated protein crystals and its comparison with X-ray data. *Proc. Natl. Acad. Sci. USA*. 80:4315–4319.

Vickerman, K. 1985. Developmental cycles and biology in pathogenic trypanosomes. *Br. Med. Bull.* 41:105–114.

Vickerman, K., and A. G. Luckins. 1969. Localisation of variable antigens in the surface coat of *Trypanosoma brucei* using ferritin-conjugated antibody. *Nature (Lond.)*. 224:1125–1127.

Wallach, M., D. Fong, and K.-P. Chang. 1982. Post-transcriptional control of tubulin biosynthesis during leishmanial differentiation. *Nature (Lond.)*. 299:650–652.