'GPEET' procyclin is the major surface protein of procyclic culture forms of *Trypanosoma brucei brucei* strain 427

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The surface of *Trypanosoma brucei brucei* insect forms is covered by an invariant protein coat consisting of procyclins. There are six or seven procyclin genes that encode unusual proteins with extensive tandem repeat units of glutamic acid (E) and proline (P) (referred to as EP repeats), and two genes that encode proteins with internal pentapeptide (GPEET) repeats. Although the EP forms of procyclins have been isolated and characterized by several laboratories, evidence for GPEET procyclin has largely been confined to the expression of its mRNA. To characterize GPEET procyclin further, we isolated the protein from *T. b. brucei* strain 427. We found that label from [^{3}H]myristic acid and [^{3}H]ethanolamine was incorporated into GPEET procyclin and we demonstrated the protein's covalent modification with a glycosylphosphatidylinositol anchor. The major form of GPEET procyclin showed an apparent molecular mass of 22–32 kDa, was susceptible to proteolytic treatment and was found to be phosphorylated. Surprisingly, our results show that GPEET procyclin represents the major form of procyclin in *T. b. brucei* 427 culture forms and that the ratio of EP to GPEET procyclin can vary considerably between different cell lines.

INTRODUCTION

African trypanosomes are transmitted between mammals by the tsetse fly. While cycling between the mammalian host and the insect vector, trypanosomes express two different types of stage-specific surface coat proteins. The surface coat of bloodstream forms of *Trypanosoma brucei brucei* consists of a dense layer of variant surface glycoproteins (VSGs) that protects the parasite from complement-mediated lysis. After bloodstream-form trypanosomes have been ingested by a tsetse fly as part of a blood meal, they differentiate into procyclic forms in the fly midgut. During this process VSG synthesis is stopped and is followed by a release of the coat from the cell surface [1–4] and its replacement by a new invariant protein coat consisting of procyclin, also called procyclic acidic repetitive protein, PARP [2,5–7]. Like the VSG in bloodstream-form trypanosomes, the procyclins and their mRNA represent some of the most abundant components in the insect form of the parasite.

Depending on the strain of *T. b. brucei*, there are six or seven procyclin genes that encode unusual proteins with extensive tandem repeat units of glutamic acid and proline (E and P respectively in the single-letter amino acid code), and two genes that encode proteins with internal pentapeptide (GPEET) repeats [5,6,8,9]. The EP forms of procyclin are closely related to each other, differing essentially in the number of EP repeats and the presence or absence of an N-glycosylation site. EP procyclins have been isolated from different strains of *T. b. brucei* by using concanavalin A lectin affinity chromatography and binding to a monoclonal antibody [7], ion-exchange chromatography followed by concanavalin A lectin affinity chromatography [10], and hydrophobic interaction chromatography [11]; it has been found that the purified proteins migrate on SDS/PAGE as a diffuse band in the apparent molecular mass range of 30–50 kDa. EP procyclin has been shown to represent the major substrate for a stage-specific, membrane-associated trans-sialidase [12,13]. By analogy with the VSGs in bloodstream-form trypanosomes [14], the EP procyclins in the insect forms have been shown to be attached to the cell surface via a glycosylphosphatidylinositol (GPI) membrane anchor [15]. The lipid and carbohydrate structures of the VSG and EP procyclin GPI anchors have been thoroughly characterized. The VSG anchor contains 1,2-dimyristoylglycerol and is substituted on its glycan core with branched side chains of α-galactose [14]; the EP procyclin anchor contains 1-stearoyl-2-*lyso*-glycerol [15] and has a large and complex carbohydrate side chain containing galactose, *N*-acetylglucosamine and sialic acid [11]. In addition the EP procyclin anchor contains a fatty acid attached to the inositol ring [15] rendering the GPI structure insensitive to cleavage by GPI-hydrolysing phospholipases C [16]. The profound structural differences in the GPI moieties of VSG and EP procyclin indicate that the *T. b. brucei* parasites not only exchange their protein surface coat during differentiation but also use different GPI structures to anchor the stage-specific coat proteins in the cell membrane.

The GPEET genes have been found to be conserved in different stocks of *T. b. brucei* [9,17]. The polypeptide precursors have the same highly conserved signal peptide and hydrophobic C-terminal peptides as the EP procyclins but the mature proteins show only very little sequence identity. The GPEET protein has only recently been identified. By using polyclonal antibodies raised against a synthetic pentapeptide repeat, GPEET procyclin was shown to be co-expressed with the EP procyclin form on the surface of *T. b. brucei* insect forms [18]. The same antibodies detected a protein with an apparent molecular mass of approx. 21 kDa on Western blots [18].

In recent work designed to study the function of the procyclins

Abbreviations used: CMW, chloroform/methanol/water; EP repeats, tandem repeat units of glutamic acid and proline; GPI, glycosylphosphatidylinositol; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; PI-PLC, phosphatidylinositol-specific phospholipase C; VSG, variant surface glycoprotein.

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in *T. b. brucei*, deletion mutants with no EP genes and only one copy of GPEET were generated. This last procyclin gene could not be deleted [18]. The EP knockouts were morphologically indistinguishable from the parental strain but showed a limited ability to establish a heavy infection in the insect midgut. These results suggest that the two forms of procyclin might have different roles: GPEET procyclin is required for cell growth in culture, whereas EP procyclin enhances survival in the fly.

To further characterize GPEET procyclin in insect-form trypanosomes, we isolated the protein from *T. b. brucei* strain 427. By using a series of cloned derivatives that express different amounts of EP procyclin, we studied the relative abundance of the GPEET form compared with the EP form in these cells and characterized its mode of membrane attachment. Our results demonstrate that GPEET procyclin is modified by a GPI anchor and, surprisingly, represents the major form of procyclin of *T. b. brucei* 427 culture forms. Furthermore we found that the ratio of EP to GPEET procyclin can vary considerably between different cell lines.

EXPERIMENTAL

Materials

Unless otherwise stated, all reagents were of A. R. grade and were from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany). [9,10-^{3}H(N)]Myristic acid ([^{3}H]myristic acid; 10–30 Ci/mmol) was purchased from DuPont–NEN (Regensdorf, Switzerland) and [1-^{3}H]ethan-1-ol-2-amine hydrochloride ([^{3}H]ethanolamine; 18–29 Ci/mmol) was from Amersham (Zürich, Switzerland). Acrylamide solution was from National Diagnostics (Kingston upon Hull, Humberside, U.K.). Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* and alkaline phosphatase from calf intestine (grade II) were purchased from Boehringer (Mannheim, Germany). GPI-specific phospholipase D (GPI-PLD) from bovine serum was purified as described elsewhere [19]. The anti-(EP procyclin) monoclonal antibody TRBP1/247 [7,20] was provided by Terry W. Pearson (University of Victoria, Victoria, BC, Canada). Polyclonal antibodies against GPEET procyclin were raised against a synthetic peptide, (GPEET)$_3$C, conjugated with keyhole limpet haemocyanin [18]. Horseradish-peroxidase-conjugated rabbit anti-mouse and pig anti-rabbit antibodies were purchased from Dako (Copenhagen, Denmark).

Trypanosomes

The parental strain *T. b. brucei* 427 [21] and the derivative clones Nour 6C and N6-EPα2A were cultured at 27 °C in SDM-79 medium containing 5 % (v/v) heat-inactivated fetal bovine serum [22]. *T. b. brucei* 427 has the full complement of procyclin genes (six copies of EP and two copies of GPEET). The deletion mutant Nour 6C has no EP genes and only one copy of GPEET; N6-EPα2A trypanosomes, which were derived from Nour 6C, have one overexpressed copy of a glycosylated form of EP in addition to the single copy of GPEET [18]. *T. b. brucei* STIB 247 (strain 247) was isolated in 1971 in the Serengeti National Park (Tanzania) from a hartebeest [23]. *T. b. brucei* STIB 348 (strain 348) is a cloned population of strain 247. *T. b. brucei* STIB 777 (strain 777) is a derivative of KETRI 1957, which was isolated in 1971 from a wild *Glossina fuscipes fuscipes* in Busoga, Uganda [24]. Strains 247, 348 and 777 were all obtained from R. Brun (Swiss Tropical Institute, Basel, Switzerland). The procyclic cell line Rhode PRO G 141287 (strain PRO G) was isolated from the midgut of *Glossina morsitans morsitans* infected with the EATRO 1125 stock of *T. b. brucei* and was obtained from E. Pays and S. Rolin (University of Brussels, Brussels, Belgium).

Labelling of cells

With myristic acid

Typically, 50 μCi of [^{3}H]myristic acid was dissolved in one drop of ethanol and added to 0.5 ml of culture medium containing 5 mg/ml defatted BSA. The suspension was subsequently added, together with 5 ml of fresh medium, to 5 ml of trypanosomes [(1–3) $\times 10^7$ cells/ml], and the cells were incubated for 16–18 h at 27 °C during which time the cell number roughly doubled.

With [^{3}H]ethanolamine

[^{3}H]Ethanolamine (50 μCi) was added, together with 5 ml of fresh medium, to the cells as described above.

Extraction of trypanosomes

Trypanosomes [(1–4) $\times 10^8$ cells] were pelleted by centrifugation (15 min, 2000 ***g***, 4 °C) and washed once with ice-cold 10 mM Tris/HCl, pH 7.4, containing 140 mM NaCl. Chloroform and methanol were added to the aqueous cell pellet (0.9 ml) to give a final ratio of chloroform/methanol/water (CMW) of 10:10:3 (by vol.); the extract was then centrifuged as above. After two additional extractions with CMW, the pellet was extracted twice with 1 ml of 9 % (v/v) butan-1-ol in water, and the butanol phases were combined (butanol extract). The insoluble material was subsequently extracted with 0.3 ml of 0.1 % (w/v) Triton X-100 in 20 mM Tris/HCl, pH 7.4, and boiled for 10 min (Triton extract). The residual pellet was solubilized by boiling for 10 min in 1 % (w/v) SDS (SDS extract). In some experiments, the pooled CMW-soluble fractions were dried under nitrogen and the residue was partitioned between butan-1-ol and water, resulting in fractions F1 (butanol-rich phase) and F2 (aqueous phase) [25]. Aliquots of all extracts were counted for radioactivity, and the extracts were stored at −20 °C.

Octyl-Sepharose chromatography

The GPI-anchored structures of the various radiolabelled extracts were isolated by chromatography on octyl-Sepharose [11,25,26]. Dried butanol extracts were resuspended in 0.3 ml of 100 mM ammonium acetate containing 5 % (v/v) propan-1-ol (buffer A) and fractionated on an octyl-Sepharose column (2 ml bed volume) previously equilibrated in buffer A. Triton and SDS extracts were applied directly to the column. The column was washed with 5 ml of buffer A followed by 5 ml of 100 mM ammonium acetate containing 20 % (v/v) propan-1-ol, and was eluted with a linear gradient of 20–40 % (v/v) propan-1-ol in 100 mM ammonium acetate. Fractions of 0.5 ml were collected; aliquots were counted for radioactivity. The actual propan-1-ol concentration in an individual fraction was determined by measuring the refractive index. Fractions of interest were pooled and stored at 4 °C.

Concanavalin A–Sepharose chromatography

^{3}H-labelled material from butanol extracts was applied to a concanavalin A–Sepharose column (2 ml bed volume) previously equilibrated in 25 mM Hepes/NaOH (pH 7.5)/150 mM NaCl/5 mM $CaCl_2$/5 mM $MgCl_2$/5 mM $MnCl_2$, containing 0.1 % (w/v) Triton X-100 (buffer B). The column was washed with 6 ml of the same buffer, and bound material was eluted with

buffer B containing 0.5 M methyl-α-D-mannopyranoside. Fractions of 0.5 ml were collected and counted for radioactivity.

Enzymic and chemical treatment of trypanosome extracts

^{3}H-labelled material from the various extracts and from fractions after octyl-Sepharose chromatography was treated with PI-PLC or GPI-PLD [26], or with nitrous acid or mild base [27] exactly as described. Released hydrophobic products were extracted with toluene or water-saturated butan-1-ol, or by a sequential combination of both. Treatment of [^{3}H]ethanolamine-labelled butanol extracts with aqueous HF was done as described before [28]. After 48 h of incubation the samples were dried in the SpeedVac and resuspended in 1 % (w/v) SDS.

TLC

One-dimensional TLC was done on Silica Gel 60 plates with chloroform/methanol/acetone/acetic acid/water (40:15:15:12:8, by vol.) [26].

Protease treatment of trypanosome extracts

[^{3}H]-labelled butanol extracts were treated with trypsin [8 μg in 50 μl of 10 mM Tris (pH 7.4)/140 mM NaCl/50 mM $CaCl_2$/0.1 % Triton X-100] or Pronase [300 μg in 30 μl of 50 mM Tris (pH 7.5)/5 mM $CaCl_2$] for 24 h at room temperature.

Treatment of cell lysates with alkaline phosphatase

Trypanosomes (10^6 cells) were incubated in the presence or absence of 10 μg of alkaline phosphatase from calf intestine (140 units/mg) in 20 μl of 25 mM diethanolamine, pH 9.8, containing 0.5 mM $MgCl_2$, for 4–16 h at 37 °C.

SDS/PAGE and Western blotting

SDS/PAGE was performed under reducing conditions [29] on 12 % (w/v) polyacrylamide gels. For autoradiography, gels were soaked in Amplify (Amersham), dried and then exposed to Kodak X-Omat S films at −70 °C. Electroblotting of proteins from polyacrylamide gels on nitrocellulose sheets with subsequent blocking of membranes with milk powder and incubation with antibodies was done as described by Hehl et al. [30]. Antigen binding was detected with an enhanced chemiluminescence detection kit (Amersham), in accordance with the instructions of the manufacturer.

Amino acid analysis

Octyl-Sepharose-purified material was subjected to amino acid analysis after 6 M HCl hydrolysis with a Hewlett–Packard 1090 automated amino acid analyser system. N-terminal amino acid sequences were determined with a pulsed liquid-phase Applied Biosystems sequencer model 477A.

RESULTS

Labelling of procyclic trypanosomes and sequential extraction of labelled material

Procyclic culture forms can be labelled with [^{3}H]myristic acid, resulting in the incorporation of radiolabel into protein [15]. We found that incubation of *T. b. brucei* 427 cells [$(0.4–1.2)\times10^8$ cells] overnight at 27 °C with 40–50 μCi of [^{3}H]myristic acid resulted in the incorporation of $(3–7)\times10^6$ c.p.m. (range of ten independent experiments) into SDS-solubilized protein after extensive delipidation with CMW. The use of smaller amounts of [^{3}H]myristic acid (10–20 μCi) considerably decreased the incorporation of radioactivity into protein, whereas larger amounts (100 μCi) did not result in higher yields. When the cells were incubated with 50 μCi [^{3}H]ethanolamine instead of [^{3}H]myristic acid, similar amounts of radioactivity (8×10^6 c.p.m.; mean of two independent experiments) were incorporated into the delipidated material.

Table 1 Enzymic and chemical treatment of [^{3}H]myristic acid-labelled extracts of *T. b. brucei* 427 procyclic cells

Results are means of single determinations from two to four separate experiments.

Treatment	Release of radiolabel (%)		
	Butanol extract	Triton extract	SDS extract
Nitrous acid	77.4	76.6	22.3
Blank*	0.6	3.9	4.1
GPI-PLD	61.9	63.3	18.6
PI-PLC	0.7	4.8	7.1
Blank†	0.5	3.8	6.3
Mild base	84.2	80.1	69.3
Blank‡	0.8	3.4	2.0

* Control samples were incubated in buffer containing NaCl instead of nitrous acid.
† Control samples were incubated in the absence of phospholipases.
‡ Control samples were incubated in ethanol containing NaCl instead of NaOH.

The incorporation of radioactivity into SDS-solubilized procyclin has been studied previously [15]. In agreement with that report we found that a [^{3}H]myristic acid-labelled SDS extract of *T. b. brucei* 427 cells contained multiple radiolabelled products (results not shown) and that 30–40 % of the radioactivity could be released by treatment with GPI-PLD, indicating the presence of GPI-anchored material. More recently a protocol was described for the purification of procyclin that involved its extraction from delipidated material with 9 % (v/v) butan-1-ol [11]. When we applied this procedure to extract radiolabelled procyclin from delipidated cell pellets, we found that only 40–60 % of total counts (range of 10 independent experiments) were extracted by water-saturated butan-1-ol. Treatment of the extracted [^{3}H]myristate-labelled material with nitrous acid and GPI-PLD released more than 75 % and more than 60 % respectively of the radioactivity (Table 1), indicating that the label in the butanol extract was incorporated into the lipid moiety of GPI-anchored structures. The complete insensitivity of the [^{3}H]myristate-labelled material to PI-PLC treatment (Table 1) is consistent with the phosphatidylinositol moiety's being acylated on the inositol ring [16]. Subsequent extraction of the butanol-insoluble residue with 0.1 % (w/v) Triton X-100 resulted in the solubilization of another 7–17 % of the total radioactivity (range of eight independent experiments). The radiolabelled material in the Triton extract showed the same sensitivity to nitrous acid, GPI-PLD and PI-PLC as that in the butanol extract (Table 1). Finally, the Triton X-100-insoluble pellet was solubilized in 1 % (w/v) SDS and was found to contain little additional radiolabelled material sensitive to nitrous acid and GPI-PLD (Table 1). When the radiolabelled reaction products released from [^{3}H]myristate-labelled butanol extracts by GPI-PLD treatment were analysed by TLC, all radioactivity co-migrated with the acyl-*lyso*-phosphatidic acid standard (results not shown). Together with the observation that the radiolabel in [^{3}H]myristate-labelled butanol extracts was completely sensitive

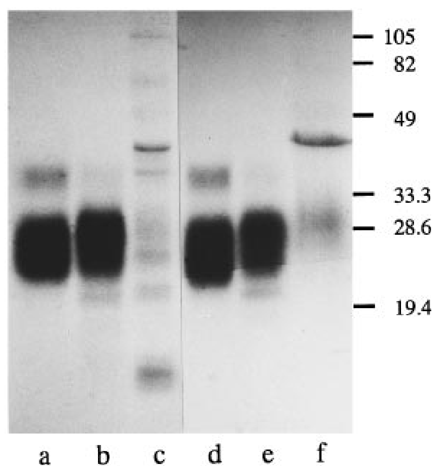

Figure 1 Labelling of *T. b. brucei* 427 extracts with myristic acid and ethanolamine

T. b. brucei 427 procyclic cells were incubated with [^{3}H]myristic acid or [^{3}H]ethanolamine for 16–18 h, washed to remove non-incorporated radiolabel and extracted sequentially with CMW followed by 9% (v/v) butan-1-ol, 0.1% (w/v) Triton X-100 and 1% (w/v) SDS. Aliquots of the extracts were analysed by SDS/PAGE and autoradiography. Lanes a–c, extracts of cells labelled with [^{3}H]myristic acid; lanes d–f, extracts of cells labelled with [^{3}H]ethanolamine; lanes a and d, butanol extracts; lanes b and c, Triton extracts; lanes c and f, SDS extracts. The lanes contained equal amounts of radioactivity. The positions of molecular-mass markers are indicated (in kDa) at the right.

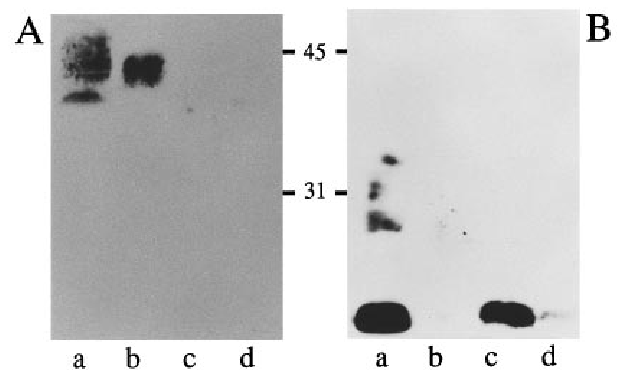

Figure 2 Identification of EP and GPEET procyclins in *T. b. brucei* 427 extracts by Western blotting

T. b. brucei 427 procyclic cells were extracted sequentially as described in the legend to Figure 1; the extracts were analysed by SDS/PAGE followed by immunoblotting with monoclonal anti-EP (**A**) and polyclonal anti-GPEET (**B**) antibodies. Lanes a, cell lysates; lanes b, butanol extracts; lanes c, Triton extracts; lanes d, SDS extracts. The lanes contained equal amounts of cells or cell equivalents. The positions of molecular-mass markers are indicated (in kDa) between the panels.

to mild base treatment (Table 1), these results are consistent with the label's being associated with GPI-anchored structures containing an inositol-acylated acyl-*lyso*-phosphatidic acid lipid moiety. A similar GPI lipid moiety has been demonstrated previously for EP procyclin [15].

Characterization of (radiolabelled) proteins in extracts of *T. b. brucei* 427 procyclic trypanosomes

Analysis of [^{3}H]myristic acid-labelled material from *T. b. brucei* 427 cell extracts by SDS/PAGE followed by autoradiography showed a variety of radiolabelled bands (Figure 1). The butanol extract contained a major broad band with a molecular mass ranging from 22 to 32 kDa and a minor band at 40–42 kDa (Figure 1, lane a). The band at 22–32 kDa, together with a fainter band at approx. 20 kDa, was also present in the Triton X-100 extract (Figure 1, lane b). The SDS extract showed the presence of multiple bands ranging from approx. 10 to more than 100 kDa (Figure 1, lane c). Identical patterns were obtained in the butanol and Triton X-100 extracts when 427 cells were labelled with [^{3}H]ethanolamine instead of [^{3}H]myristic acid (Figure 1, lanes d and e), whereas the [^{3}H]ethanolamine-labelled SDS extract showed a predominant labelled band at approx. 45 kDa (Figure 1, lane f). Although we have not pursued it further, this protein is most likely to be translation elongation factor EF-1α, which can be labelled with ethanolamine in a variety of cell types [31]. In some experiments the pooled CMW extracts were dried and partitioned between butan-1-ol and water, resulting in fractions F1 (butanol-rich phase, containing the lipids) and F2 (aqueous phase). Analysis of fraction F2 by SDS/PAGE followed by autoradiography showed a similar labelling pattern to that of the butanol extract (results not shown), indicating that some of the labelled protein material was extracted into CMW.

The presence of the EP and GPEET forms of procyclin in cell lysates and extracts was studied by using specific antibodies against EP [7] and GPEET. When Western-blot analysis was performed with lysates of *T. b. brucei* 427 cells, the anti-EP antibody bound to protein that migrated with an apparent molecular mass of approx. 42 kDa (Figure 2A, lane a), whereas the anti-GPEET antibody predominantly recognized a protein of approx. 20 kDa and, to a smaller extent, some material migrating at approx. 30 kDa (Figure 2B, lane a). When we probed the different cell extracts with the anti-EP antibody, we found that EP procyclin was almost exclusively present in the butanol extract (Figure 2A, lane b). In contrast, the use of the anti-GPEET antibody demonstrated the selective presence of the 20 kDa form of GPEET procyclin in the Triton X-100 extract (Figure 2B, lane c).

Together these results suggest that the EP and GPEET forms of procyclin as recognized by specific antibodies partition preferentially into the butanol and Triton X-100 extracts respectively of delipidated procyclic trypanosome lysates after sequential extraction with 9% (v/v) butan-1-ol followed by Triton X-100 and SDS. Interestingly, however, most of the radioactivity present in delipidated cell pellets after incubation of trypanosomes with [^{3}H]myristic acid or [^{3}H]ethanolamine was not associated with either known form of procyclin but with a previously unrecognized GPI-anchored structure of apparent molecular mass 22–32 kDa.

Characterization of radiolabelled structures in extracts of Nour 6C and N6-EPα2A cells

Nour 6C trypanosomes are deletion mutants that have no EP genes but still express similar amounts of GPEET procyclin when compared with *T. b. brucei* 427 cells [18]. N6-EPα2A trypanosomes were generated by reintroduction of an EP procyclin gene into Nour 6C cells, resulting in a 3-fold overexpression of EP mRNA relative to 427 cells [18]. When Nour 6C and N6-EPα2A cells were labelled with [^{3}H]myristic acid, followed by sequential extraction of the delipidated cell pellets with 9% (v/v) butan-1-ol, Triton X-100 and SDS, the radiolabelled material in the different extracts was found to have the same sensitivity to nitrous acid, GPI-PLD and PI-PLC treatment as that from extracts of wild-type cells (results not shown). Analysis

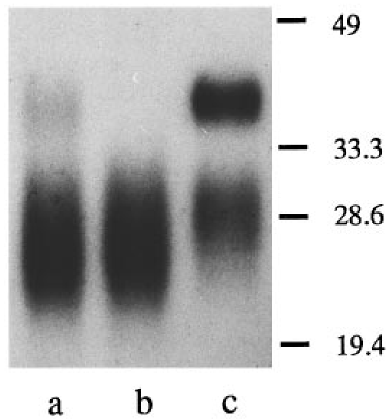

Figure 3 Labelling of *T. b. brucei* 427 mutant cells with myristic acid

Procyclic cells were incubated with [^{3}H]myristic acid and extracted sequentially as described in the legend to Figure 1. Aliquots of the butanol extracts of *T. b. brucei* 427 wild-type strain (lane a), EP-deficient Nour 6C cells (lane b) and EP-overproducing N6-EPα2A cells (lane c) were analysed by SDS/PAGE and autoradiography. The lanes contained equal amounts of radioactivity. The positions of molecular-mass markers are indicated (in kDa) at the right.

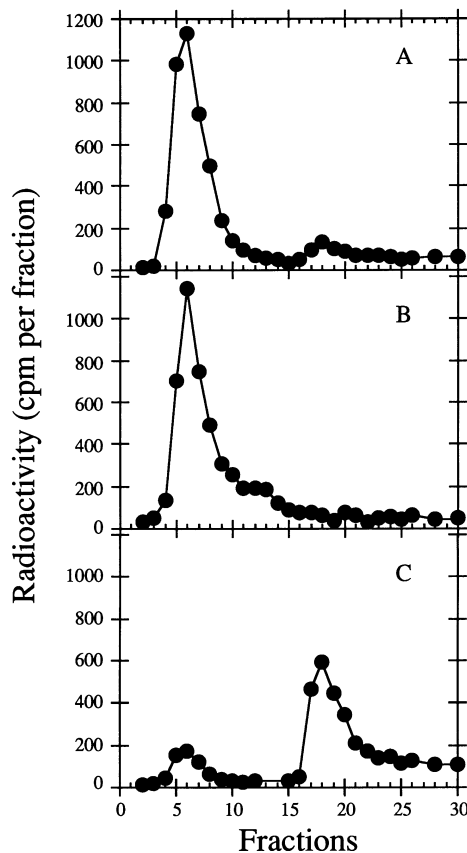

Figure 4 Concanavalin A–Sepharose chromatography of butanol extracts from *T. b. brucei* mutant cells

Procyclic cells were incubated with [^{3}H]myristic acid and extracted sequentially as described in the legend to Figure 1. Aliquots of the butanol extracts of *T. b. brucei* 427 wild-type strain (**A**), EP-deficient Nour 6C cells (**B**) or EP-overproducing N6-EPα2A cells (**C**) were applied to a concanavalin A–Sepharose column. The column was washed (fractions 1–15) and bound material was eluted with buffer containing methyl-α-D-mannopyranoside. Equal amounts of radioactivity were applied to each column and the total fractions were counted in a scintillation counter.

of the radiolabelled material by SDS/PAGE followed by autoradiography showed that the butanol extracts of 427, Nour 6C and N6-EPα2A cells all possessed the broad band at 22–32 kDa (Figure 3, lanes a–c). The 40–42 kDa band in 427 cells (Figure 3, lane a) was absent from the EP-negative Nour 6C cells (Figure 3, lane b), whereas it was heavily labelled in the EP-overexpressing N6-EPα2A trypanosomes (Figure 3, lane c). The [^{3}H]myristic acid labelling patterns of the Triton X-100 and SDS extracts of Nour 6C and N6-EPα2A cells were similar to those of 427 cells (results not shown). Furthermore the anti-EP antibody showed no reaction with cell lysates or extracts of Nour 6C trypanosomes, whereas the distribution of anti-EP- and anti-GPEET-reactive material in the sequential extracts of N6-EPα2A cells was similar to that in *T. b. brucei* 427 cells (results not shown).

Concanavalin A–Sepharose chromatography of [^{3}H]myristic acid-labelled butanol extracts

It has been previously shown that EP procyclin binds to concanavalin A [7,10], whereas GPEET procyclin contains no potential N-linked glycosylation sites [9] and thus is expected not to bind. When we applied aliquots of a [^{3}H]myristic acid-labelled butanol extract from *T. b. brucei* 427 cells to a concanavalin A–Sepharose column, we found that most of the radiolabelled material (80–85 % of the recovered radioactivity) ran in the flow-through, whereas a small fraction of the label bound to concanavalin A and was eluted from the column after addition of methyl-α-D-mannopyranoside (Figure 4A, fractions 15–25). In contrast, [^{3}H]myristic acid-labelled material from the butanol extract of Nour 6C cells was recovered exclusively in the flow-through of the column (Figure 4B), whereas most material of N6-EPα2A cells was found to bind to concanavalin A–Sepharose (Figure 4C). These results are consistent with most of the [^{3}H]myristate-labelled material in the butanol extract of *T. b. brucei* 427 cells' being incorporated into structures that, unlike the previously characterized major form of EP procyclin, do not bind to concanavalin A.

Proteolytic treatment of ^{3}H-labelled butanol extracts

To exclude the possibility that the radiolabel was associated with non-protein material (i.e. complex GPI lipids or GPI-linked polysaccharides; see [32,33] for recent reviews), we treated aliquots of ^{3}H-labelled butanol extracts with proteases and analysed the reaction products by SDS/PAGE followed by autoradiography (Figure 5). Our results showed that trypsin treatment of a Nour 6C butanol extract only slightly decreased the apparent molecular mass of the band at 22–32 kDa (Figure 5, lanes a and b), whereas treatment with Pronase resulted in its decreasing to 17–22 kDa (Figure 5, lanes c and d). A similar band was obtained when aliquots of N6-EPα2A extracts were treated with Pronase (results not shown). These results clearly indicate that the radiolabel in the butanol extracts was associated with protein. Interestingly, however, despite the use of a large excess of proteases, the radiolabelled band was not degraded to low-molecular-mass products migrating at or near the front of the gel but migrated as a broad band with only a slightly decreased apparent molecular mass compared with the untreated protein. These results indicate that the treated material is either resistant to further proteolytic degradation or alternatively consists of non-protein structures.

HF treatment of [^{3}H]ethanolamine-labelled butanol extracts

The GPI core structure contains two phosphodiester bonds that can be hydrolysed by aqueous HF [28,34]; we used this treatment

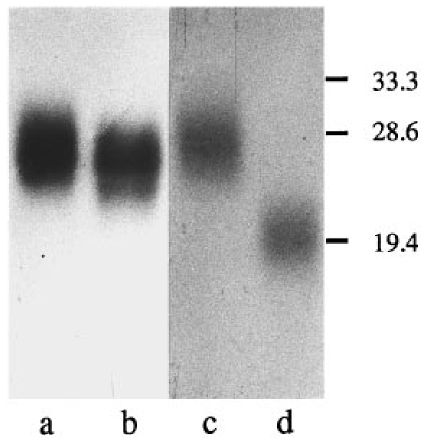

Figure 5 Protease treatment of *T. b. brucei* butanol extracts

Procyclic cells were incubated with [^{3}H]myristic acid and extracted sequentially as described in the legend to Figure 1. Aliquots of the butanol extract of *T. b. brucei* Nour 6C cells were incubated for 24 h in the absence (lanes a and c) or presence of large amounts of trypsin (lane b) or Pronase (lane d) and analysed by SDS/PAGE and autoradiography. The lanes contained equal amounts of radioactivity. The positions of molecular-mass markers are indicated (in kDa) at the right.

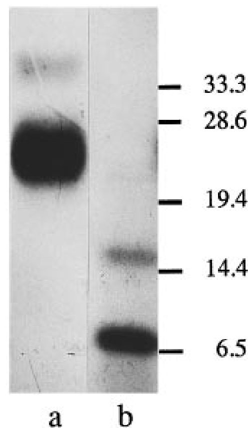

Figure 6 HF treatment of *T. b. brucei* butanol extracts

T. b. brucei 427 cells were incubated with [^{3}H]myristic acid and extracted sequentially as described in the legend to Figure 1. Aliquots of the butanol extract were incubated for 48 h in the absence (lane a) or presence (lane b) of aqueous HF, dried and then analysed by SDS/PAGE and autoradiography. The positions of molecular-mass markers are indicated (in kDa) at the right.

to release the GPI anchor from [^{3}H]ethanolamine-labelled protein from *T. b. brucei* 427 butanol extracts. Our results showed that HF treatment decreased the apparent molecular masses of the two radiolabelled bands at 40–42 kDa and 22–32 kDa to approx. 16 kDa and 7 kDa respectively (Figure 6). The observation that the [^{3}H]ethanolamine-labelled band at 22–32 kDa runs much more compactly after HF treatment indicates that the heterogeneity in the molecule was removed by HF.

Isolation and amino acid analysis of a novel GPI-anchored protein from *T. b. brucei* 427 cells

Our results so far have shown that *T. b. brucei* 427 procyclic cells, as well as Nour 6C and N6-EPα2A trypanosomes, incorporate large amounts of [^{3}H]myristic acid and [^{3}H]ethanolamine into a previously unnoticed GPI-anchored protein. The protein migrates on SDS/PAGE with an apparent molecular mass of 22–32 kDa, i.e. between the GPEET and EP forms of the procyclins as recognized by specific antibodies, and is partly susceptible to the action of proteases. To characterize the novel protein we purified it from the butanol extract of EP-deficient Nour 6C cells by octyl-Sepharose chromatography. This procedure has been applied successfully to the purification of EP procyclin from *T. brucei* 427 cells, resulting in an essentially pure preparation of the protein [11]. Chromatography of [^{3}H]myristic acid- or [^{3}H]ethanolamine-labelled butanol extract from Nour 6C cells on an octyl-Sepharose column revealed a single peak of radioactivity that was eluted at 22–24 % (v/v) propan-1-ol (results not shown). The average yield of octyl-Sepharose-purified protein from the butanol extract of Nour 6C cells was 147 μg per 10^{10} cells (mean of three experiments). Amino acid analysis of the peak material showed that it contained a protein rich in Gly, Glx and Pro residues (Table 2). This composition was similar to, but not identical with, the predicted amino acid composition of the GPEET form of procyclin, and was clearly different from that of EP procyclin (Table 2). Amino acid sequence analysis of octyl-Sepharose-purified Nour 6C butanol extract, however, revealed a N-terminal sequence of 19 amino acid residues that was identical with the stretch between residues 12 and 30 of the predicted GPEET procyclin sequence (Table 3). In addition we noted the presence of three minor sequences in the purified extract all corresponding to internal sequences of GPEET procyclin (Table 3).

In contrast, when [^{3}H]ethanolamine-labelled butanol extract was treated with nitrous acid before octyl-Sepharose chromatography, all radioactivity was eluted in the flow-through of the column (results not shown). This finding is consistent with the removal by nitrous acid of the hydrophobic moiety of the protein responsible for interaction with the column material. Further-

Table 2 Amino acid composition of [^{3}H]myristate-labelled N6 butanol extract after purification by octyl-Sepharose chromatography

Results are means ± S.D. for four separate experiments.

	Composition (%)		
Amino acid	Butanol extract	GPEET procyclin*	EP procyclin†
Asx	3.1 ± 0.8	4.6	6.2
Glx	38.6 ± 2.9	33.9	34.4
Ser	1.0 ± 0.3	3.1	
Gly	21.2 ± 2.3	16.9	10.4
His	0.9 ± 0.8		
Arg	2.7 ± 0.7	1.5	
Thr	6.7 ± 0.3	10.8	4.2
Ala	0.2 ± 0.2	3.1	2.1
Pro	19.4 ± 1.0	16.9	34.4
Tyr	0.6 ± 0.5		
Val	1.1 ± 0.6	3.1	1.0
Met	0.2 ± 0.2		
Ile	0.2 ± 0.2	1.5	
Leu	0.3 ± 0.2		1.0
Phe	0.1 ± 0.1		
Lys	3.8 ± 0.6	4.6	6.3

* Theoretical value based on the predicted GPEET procyclin sequence (amino acids 28–92 of the precursor) [9].

† Theoretical value based on the predicted EP procyclin sequence (amino acids 28–123 of the precursor) [6].

Table 3 Amino acid sequence analysis of octyl-Sepharose-purified N6 butanol extract

The sequence labelled GPEET is the amino acid sequence of the predicted GPEET procyclin sequence [9]. The N-terminal amino acid was based on the result of the N-terminal amino acid sequence analysis of EP procyclin [7]. The sequence labelled 'major sequence' is the result of a single experiment; the sequence shown was confirmed in a separate experiment with material from a different cell extract. In addition, the sequence of the first 11 amino acids was confirmed once more in a third experiment. The three minor sequences were identified in addition to the major sequence shown above; together they represent less than 10% of the total material. Abbreviation: x, unidentified amino acid.

		1 20 40 60
GPEET		ADESASNVIVKGGKGKEREDGPEEPEETGPEETGPEETGPEETGPEETGPEETGPEETEPEPEPG
Major sequence		GGKGKEREDGPEEPEExGP
Minor sequence	1	KGGKG
	2	VKGGK
	3	EDGPE

more, analysis of the column fractions of the nitrous acid-treated extract that was eluted at 22–24% propan-1-ol showed only negligible (i.e. background) quantities of amino acids, indicating that the analyses shown in Table 2 were not significantly contaminated by non-GPI-linked protein material.

Analysis of the amino acid composition of the octyl-Sepharose-purified material after Pronase treatment of a Nour 6C butanol extract showed the predominant presence of Gly residues (approx. 80% of total amino acids) besides small amounts of Pro, Glx and Asx (each less than 6% of total amino acids). This finding is consistent with an efficient proteolytic degradation by Pronase of GPEET procyclin down to the last amino acid of the predicted sequence of the mature protein (Gly-92), which in common with EP procyclin [10] might represent the site of GPI anchor attachment.

Treatment of trypanosome lysates with alkaline phosphatase

Our results so far had identified the major radiolabelled protein in *T. b. brucei* extracts as a form of GPEET procyclin. However, it was unclear why our antibody raised against a synthetic GPEET pentapeptide repeat recognized only a band at approx. 20 kDa but not the more abundant and heavily labelled protein at 22–32 kDa. The (E)TGPE(E) motifs in the GPEET repeat units represent potential phosphorylation sites (i.e. for casein kinase II; underlined amino acid residues are essential, and those in parentheses stimulate phosphorylation [35]). We therefore tested whether the poor reactivity of the antibody might be due to the phosphorylation of the GPEET epitope against which it was raised. This indeed proved to be so, because the reactivity of the anti-GPEET antibody with *T. b. brucei* cell lysates was markedly increased after treatment of the cells with alkaline phosphatase (Figure 7). In control cells incubated in the absence of alkaline phosphatase, the antibody recognized mainly a protein of approx. 20 kDa (Figure 7, lane a), whereas in cells treated with alkaline phosphatase it reacted strongly with a broad band migrating at 20–32 kDa and, in addition, recognized a band at approx. 19 kDa (Figure 7, lane b). These results also explain the irregular antibody staining pattern seen in Figure 2(B), lane A.

Relative abundance of the EP and GPEET forms of procyclin in various strains of cultured *T. b. brucei* procyclic cells

To study the presence of GPEET procyclin in other strains of cultured procyclic trypanosomes we labelled four additional strains of *T. b. brucei* with [^{3}H]myristic acid and analysed the distribution of radioactivity between the EP and GPEET forms of procyclin in the butanol extracts. Our results showed that, although the incorporation of radioactivity into all four strains was indistinguishable from *T. b. brucei* 427 and its derivative cell lines, the relative distribution of radioactivity between EP and GPEET procyclin varied considerably (Figure 8). Strains 247

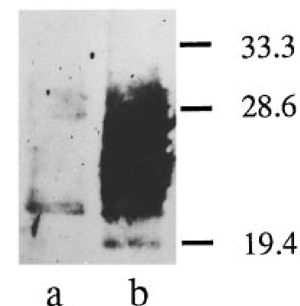

Figure 7 Treatment of trypanosomes with alkaline phosphatase

T. b. brucei cells (10^6 cells) were incubated in the absence (lane a) or presence (lane b) of alkaline phosphatase for 16 h at 37 °C. At the end of the incubation, the control sample was supplemented with heat-inactivated alkaline phosphatase and the total samples were analysed by SDS/PAGE followed by immunoblotting with polyclonal anti-GPEET antibodies. The positions of molecular mass markers are indicated (in kDa) at the right.

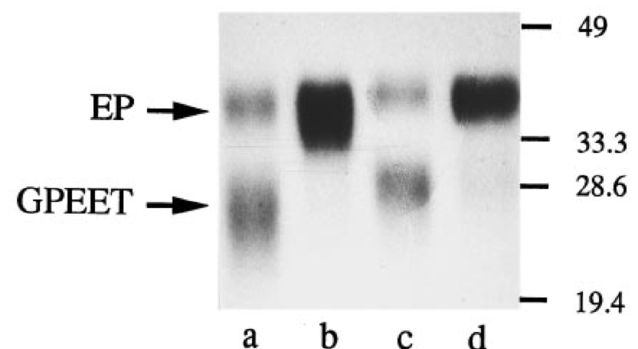

Figure 8 Labelling of various procyclic strains with myristic acid

Procyclic cells were incubated with [^{3}H]myristic acid and extracted sequentially as described in the legend to Figure 1. Aliquots of the butanol extracts of *T. b. brucei* strains 247 (lane a), 777 (lane b), PRO G (lane c) and 348 (lane d) were analysed by SDS/PAGE and autoradiography. The lanes contained equal amounts of radioactivity. The positions of molecular-mass markers are indicated (in kDa) at the right.

(Figure 8, lane a) and PRO G (Figure 8, lane c) showed the presence of large amounts of labelled GPEET procyclin besides smaller amounts of labelled EP procyclin, whereas in strains 777 (Figure 8, lane b) and 348 (Figure 8, lane d) only labelled EP procyclin was detected at this exposure.

DISCUSSION

The EP procyclins are major surface proteins of procyclic forms of *T. b. brucei* and have been isolated and characterized by several laboratories [7,10,11]. In contrast, proof for the expression of the GPEET form of procyclin has largely been confined to the presence of its mRNA [9]. Only very recently, GPEET procyclin has been identified in procyclic trypanosomes by immunofluorescence staining and Western-blot analysis with an antibody raised against the GPEET pentapeptide repeat [18].

It has been shown previously that procyclic *T. b. brucei* 427 cells can be labelled with [^{3}H]myristic acid and [^{3}H]ethanolamine [10,15]. The bulk of radioactivity was found to be incorporated into a prominent broad band at approx. 45 kDa apparent molecular mass, and it was shown that the label was associated with the GPI anchor of the EP form of procyclin. Surprisingly, when we labelled *T. b. brucei* 427 cells with [^{3}H]myristic acid or [^{3}H]ethanolamine we found a distinctly different labelling pattern from those published previously [10,15]. Most of the label (more than 80 %) was incorporated into a broad band migrating with an apparent molecular mass of 22–32 kDa, whereas the remainder was associated with a band at 40–42 kDa. The susceptibility of the radiolabelled material to GPI-PLD, nitrous acid and mild base, together with its insensitivity to PI-PLC, and the mobility of the GPI-PLD reaction products on TLC, was consistent with the label's being associated with an inositol-acylated acyl-*lyso*-phosphatidic acid GPI lipid moiety. A similar lipid structure has been identified previously in the GPI anchor of EP procyclin [15]. Furthermore we found that the prominent GPI-anchored band at 22–32 kDa was present not only in *T. b. brucei* 427 cells but also in EP-deficient Nour 6C and EP-overproducing N6-EPα2A cells, indicating that its presence did not depend on the levels of EP procyclin expression. However, the label was clearly associated with protein, as demonstrated by its sensitivity to proteases. Interestingly, by using specific antibodies against the EP and GPEET forms of procyclins we found that EP procyclin was preferentially extracted from delipidated cell pellets by 9 % (v/v) butan-1-ol, whereas the approx. 20 kDa immunoreactive form of GPEET procyclin was largely resistant to butanol extraction and was soluble only in 0.1 % Triton X-100. However, the radiolabelled band at 22–32 kDa was clearly present in both the butanol and the Triton extract and its apparent molecular mass did not coincide with that of either the EP or the immunoreactive GPEET form of procyclin.

To characterize the labelled material further, we purified it from the butanol extract of Nour 6C cells and analysed its amino acid composition and partial N-terminal amino acid sequence. Our results clearly identified the protein as a form of GPEET procyclin. The major N-terminal amino acid sequence corresponded to the stretch of amino acids between residues 12 and 30 of the predicted GPEET procyclin sequence [9]. Additional minor sequences started at residues 10, 11 and 19 of the GPEET procyclin sequence, indicating that differently processed forms of GPEET procyclin might be expressed simultaneously, or alternatively that the protein is susceptible to N-terminal proteolytic processing *in vivo* or during the extraction procedure.

Treatment of radiolabelled GPEET procyclin with Pronase decreased its apparent molecular mass by approx. 7 kDa. The observation that the amino acid composition of the purified Pronase-treated material consisted almost exclusively of Gly indicated that Pronase completely degraded GPEET procyclin and confirmed that the Gly residue at position 92 of the precursor sequence represents the predicted site of GPI anchor addition (see [9]). The observed decrease in apparent molecular mass of GPEET procyclin after Pronase treatment is in good agreement with a theoretical minimal mass for GPEET procyclin of 5.7–6.8 kDa as calculated from the amino acid sequence of the protein (the mass value varies depending on the length of the GPEET procyclin sequence used for calculation). In addition, an apparent molecular mass of approx. 7 kDa for GPEET procyclin was also obtained after the removal of the GPI anchor from the protein by HF treatment. The slight decrease in apparent molecular mass of GPEET procyclin seen after trypsin treatment is consistent with the presence of potential trypsin cleavage sites close to the N-terminus of the major sequence (i.e. residues at positions 3 and 5 of the major identified amino acid sequence), resulting in the removal of only small peptides from the protein.

The observation that the Pronase-treated fragment, i.e. the GPI anchor with a single Gly residue attached to it, migrated on SDS/PAGE as a broad band with a much higher apparent molecular mass than expected (17–22 kDa compared with approx. 7 kDa) indicates that the unusual electrophoretic mobility and apparent heterogeneity of GPEET procyclin is caused by the attachment of the GPI anchor. A similar atypical behaviour on SDS/PAGE has also been observed for EP procyclin. Although the protein has a theoretical molecular mass of approx. 11.5 kDa (on the basis of the amino acid composition of the EP1 sequence and including a short carbohydrate side chain; [36]), the GPI-anchored form migrates as a broad band with an apparent molecular mass of 30–50 kDa [7,10,11,15]. The structure of the GPI anchor of EP procyclin has been analysed in detail and contains a large and complex carbohydrate side chain [11] that might be responsible for the atypical electrophoretic mobility of EP procyclin on SDS/PAGE. Interestingly, very recent data from the same laboratory indicate that the structure of the GPI anchor of GPEET procyclin is very similar to that of EP procyclin [36].

Interestingly, we found that the 22–32 kDa form of GPEET procyclin was hardly recognized by our antibody against a synthetic GPEET pentapeptide repeat. However, when cell extracts were treated with alkaline phosphatase before immunoblotting, the anti-GPEET antibody reacted strongly with the major form of GPEET procyclin, indicating that native GPEET in procyclic cells is phosphorylated and that the presence of the phosphate residues interferes with antibody binding. The presence of seven potential threonine phosphorylation sites in the GPEET procyclin sequence suggests that the protein might be phosphorylated at multiple sites. Although no attempts were made to identify the individual phosphorylated threonine residues, we propose that at least the threonine residue at position 17 of the major identified amino acid sequence is phosphorylated because no amino acid could be identified during this step of the amino acid sequence analysis. The state of phosphorylation of GPEET procyclin might also affect its extractability from delipidated cell pellets. It is possible that the presence of one or more extra phosphate residues on the 22–32 kDa form of GPEET procyclin renders the material sufficiently hydrophilic to be extracted by 9 % (v/v) butan-1-ol, whereas the approx. 20 kDa non-phosphorylated form is resistant to butanol extraction and is solubilized only by 0.1 % (w/v) Triton X-100.

Our present work was done with the same culture-adapted procyclic cell line, i.e. *T. b. brucei* strain 427, as the work by Field et al. [15]. However, whereas they reported the exclusive in-

corporation of [^{3}H]myristic acid into EP procyclin, we found that most of the radiolabel was incorporated into GPEET procyclin. Because in our cells GPEET procyclin is expressed in copy numbers similar to those reported earlier for EP procyclin [11], we conclude that GPEET procyclin represents the major form of procyclin in our *T. b. brucei* 427 cell line. At present it is not clear why these cells have switched the expression of the major surface coat protein from EP to GPEET procyclin. Interestingly, we noted that also other procyclic cell lines in culture express variable levels of EP and GPEET procyclin. Strains 777 and 348 express EP procyclin almost exclusively, whereas strains 247 and PRO G express predominantly GPEET procyclin. Because the strains 777 and 348 have been recently transmitted through tsetse flies (R. Brun, personal communication), it is possible that the expression of GPEET procyclin increases with the time for which the cells are maintained in culture.

These observations might also have direct implications for a variety of studies on the surface properties of procyclic trypanosomes. Our demonstration that the relative amounts of EP and GPEET procyclin in cultured *T. b. brucei* cells can vary considerably depending on the strain used and, possibly, on the culture time makes it essential to determine the type of procyclin expressed, because the results might change with the relative abundance of the two forms.

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