The GPI-Phospholipase C of *Trypanosoma brucei* Is Nonessential But Influences Parasitemia in Mice

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Abstract. In the mammalian host, the cell surface of *Trypanosoma brucei* is protected by a variant surface glycoprotein that is anchored in the plasma membrane through covalent attachment of the COOH terminus to a glycosylphosphatidylinositol. The trypanosome also contains a phospholipase C (GPI-PLC) that cleaves this anchor and could thus potentially enable the trypanosome to shed the surface coat of VSG. Indeed, release of the surface VSG can be observed within a few minutes on lysis of trypanosomes in vitro. To investigate whether the ability to cleave the membrane anchor of the VSG is an essential function of the enzyme in vivo, a GPI-PLC null mutant trypanosome has been generated by targeted gene deletion. The mutant trypanosomes are fully viable; they can go through an entire life cycle and maintain a persistent infection in mice. Thus the GPI-PLC is not an essential activity and is not necessary for antigenic variation. However, mice infected with the mutant trypanosomes have a reduced parasitemia and survive longer than those infected with control trypanosomes. This phenotype is partially alleviated when the null mutant is modified to express low levels of GPI-PLC.

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1. Abbreviations used in this paper: CRD, cross-reacting determinant; GPI, glycosylphosphatidylinositol; mf, hydrophobic membrane form; PI, phosphatidylinositol; PLC, phospholipase C; VSG, variant surface glycoprotein.

African trypanosomes are unusual among protozoan parasites in that they never enter host cells and yet persist for extended periods of time in mammalian blood and tissues. They are able to survive in the host because conserved cell surface polypeptides are shielded from host antibodies by a dense monolayer of a single polypeptide species, the variant surface glycoprotein (VSG).1 The parasites are able to establish a persistent infection by a system of antigenic variation, whereby a single trypanosome stops expressing one VSG gene and starts expressing another antigenically distinct VSG. The VSG is a homodimer, each monomer consisting of an NH₂-terminal domain comprising ~350 residues and a COOH-terminal domain(s) of 50 to 100 residues (Johnson and Cross, 1979). The structure of the NH₂-terminal domain is known in the case of two VSGs (Blum et al., 1993) and shows a high degree of conservation of tertiary structure but not primary sequence. The VSG is anchored in the membrane by the covalent attachment of the COOH-terminal carboxyl to a glycosylphosphatidylinositol (GPI), the structure of which was first solved for the VSG MITat 1.4 (Ferguson et al., 1988). The GPI anchor is added to the VSG within 1 min of the completion of protein synthesis (Ferguson et al., 1986).

*Trypanosoma brucei* contains an endogenous phospholipase C (PLC) known as the GPI-PLC (Bülow and Overath, 1986; Fox et al., 1986; Hereld et al., 1986) that is capable of hydrolyzing the GPI anchor on the VSG, releasing dimyristyl glycerol (Ferguson et al., 1985). The result of this hydrolysis is to convert the hydrophobic membrane form VSG (mVSG) to a water soluble VSG (sVSG; Cardoso de Almeida and Turner, 1983), and hypotonic lysis (Cardoso de Almeida and Turner, 1983) or stress (Voorheis et al., 1982; Rolin et al., 1996) results in the shedding of the VSG from the membrane. This conversion can be detected immunologically as it results in the exposure of the cross-reacting determinant (CRD; Holder and Cross, 1981; Cardoso de Almeida and Turner, 1983), an epitope contained in the residue of the anchor left attached to the VSG after PLC hydrolysis. Consequently mVSG is poorly recognized by anti-CRD antibodies when compared with sVSG. As well as the VSG, the majority of the precursors of the GPI anchor are also substrates for the GPI-PLC in...
vitro (for review of GPI biosynthesis see McConville and Ferguson, 1993). Recently, it has been shown that *T. brucei* GPI-PLC can hydrolyze phosphatidylinositol (PI) under certain assay conditions (Bütikofer et al., 1996). However the *Kₘ* for PI, 37 μM, is one to two orders of magnitude higher than that for GPI-linked proteins (0.4–0.7 μM for VSG [Bülow and Overath, 1986; Mensa-Wilmot and England, 1992], 0.8 μM for acetyl choline esterase [Bütikofer et al., 1996]), so, at equivalent substrate concentrations, the GPI-PLC would preferentially cleave GPI over PI, at least in vitro. It is therefore worth noting that the number of VSG and PI molecules per trypanosome is in fact roughly equivalent at 1.13 × 10⁶ molecules of the VSG (Jackson et al., 1985) and ~2.7 × 10⁷ molecules of PI (Voorheis and Martin, 1980; Carroll and McCrorie, 1986). However, the PLC has been shown to cleave both GPI and PI has added to the confusion concerning its function.

If GPI-PLC acts on a single GPI-anchored protein, then the VSG is the most obvious candidate. The VSG is by orders of magnitude the most abundant GPI-anchored protein in bloodstream trypanosomes, yet at ~3 × 10⁴ molecules of GPI-PLC/t/trypanosome (Hereld et al., 1986) and at an estimated turnover rate of 100 to 700 mVSG molecules/min under assay conditions (Hereld et al., 1986; Mensa-Wilmot and Englund, 1992), there is sufficient GPI-PLC to release the entire surface coat in a few minutes, as occurs on hypotonic or detergent lysis of trypanosomes. The question that arises is whether the GPI-PLC acts on the VSG at a point other than lysis. A low rate of sVSG release from trypanosomes has been observed in cultures of bloodstream forms and clearly demonstrated not to result from lysis of a subset of the population (Bülow et al., 1989b). In addition, release of sVSG can be induced under stress conditions that do not lyse trypanosomes (Rolin et al., 1996). Both of these observations suggest that GPI-PLC acts on mVSG in living trypanosomes, not just on cell lysis.

Furthermore, VSG and GPI-PLC show the same developmentally regulated expression, being found in bloodstream but not in procyclic trypanosomes, i.e., the form that is present in the insect midgut (Bülow and Overath, 1985; Carrington et al., 1989; Mensa-Wilmot et al., 1990). GPI-PLC expression also coincides with GPI-anchor sensitivity to GPI-PLC cleavage; all GPI anchors analyzed to date in procyclic trypanosomes are GPI-PLC resistant (Field et al., 1991; Engstler et al., 1992). In the case of the major surface protein, procyclin, this is due to palmitoylation of the inositol ring (Field et al., 1991).

The main evidence against mVSG constituting the in vivo substrate for GPI-PLC is its membrane topology. GPI-PLC acts as an integral membrane protein, despite lacking any predicted membrane spanning sequences (Carrington et al., 1989; Mensa-Wilmot et al., 1990) and appears to be predominantly localized to the cytoplasmic face of intracellular vesicles (Bülow et al., 1989a). The VSG and GPI-PLC must be in the same membrane for a reaction to occur (Bülow and Overath, 1986) and presumably also orientated on the same side of the membrane. So, given their location in the cell and without a mechanism for translocating the enzyme across a membrane, GPI-PLC could not gain access to mVSG.

The ability of the GPI-PLC to catalyze the shedding of the VSG coat in vitro, and the cotemporal expression of the two proteins, has led to models that suggest an important role for the enzyme in the developmental changes that involve alterations in expression of cell surface proteins (Carrington et al., 1991). There are three circumstances when a trypanosome must replace its surface VSG and therefore when a specific VSG-shedding activity, such as GPI-PLC, might be invoked. The first occurs within a few days after entry into a mammalian host when all cells exchange the metacyclic VSG, expressed in the insect salivary gland, for a bloodstream form VSG (Esser and Schoenbechler, 1985). The second occasion may arise in the individual trypanosomes undergoing antigenic variation. In both cases, a passive exchange of new for old VSG, based on measurements of VSG turnover (Bülow et al., 1989b) in combination with growth and dilution, would take several generations, so a process for more rapid exchange has obvious advantages in a host that is mounting an immune response to the old VSG. As antigenic variation occurs in a very small fraction of the population, it has not been possible to determine whether this occurs. The third is on the differentiation of bloodstream to procyclic trypanosomes that occurs on ingestion by the insect vector and involves the replacement of surface VSG with procyclin (Roditi et al., 1989). Although the increase in rate of VSG shedding on this differentiation can be fully explained by the activation or induction of a specific pro tease (Ziegelbauer et al., 1993), the fate of the GPI-linked, COOH-terminal peptide remains unresolved.

Other biological substrates have been suggested for the GPI-PLC. One or more of the GPI-PLC-sensitive GPI precursors is an attractive proposition since a number are found predominantly in the cytoplasmic leaflet of the endoplasmic reticulum (Vidugiriene and Menon, 1993; Mensa-Wilmot et al., 1994) and are thus topologically available to the GPI-PLC. It has been suggested that *T. brucei* GPI-PLC may play a role in the regulation of GPI levels (Güther et al., 1994; Güther and Ferguson, 1995) or PI levels (Bütikofer et al., 1996) within the cell. However, it is not immediately obvious why a general role in (G)PI metabolism or homeostasis, unlike expression of GPI-PLC and VSG, would be confined to the bloodstream stage of the life cycle.

Most of the circumstantial evidence outlined above thus points to a role for the GPI-PLC in the hydrolysis of the GPI anchor of the VSG. To address directly the role of this, a GPI-PLC null mutant (PLC⁻) has been generated and its phenotype studied. The GPI-PLC is not an essential activity, but PLC⁻ trypanosomes display an altered persistent infection in mice.

**Materials and Methods**

**Construction of the Chimeric Selectable Marker Genes**

The *neo*³ and *hyg*⁴ antibiotic resistance genes were constructed to a standard format. The procyclin mini-exon acceptor site and *S* untranslated region (UTR) was followed by the coding sequence of the resistance gene and then the 3' UTR and polyadenylation site of a *T. brucei* β-tubulin gene. The constructs were derived from pJF6 (Clayton et al., 1990). The chloramphenicol acetyl transferase gene was removed by HindIII–BamHI.
digestion and replaced, in a three-fragment ligation, by a HindIII–EcoRI fragment containing the coding sequence and an EcoRI–BglII fragment from the β-tubulin gene. The restriction sites were added to the coding sequence using PCR or adaptors.

**PLC Gene Deletion Constructs, pLN and pSH**

The gene encoding GPI-PLC (PLC) and flanking DNA was subcloned from ABS2 (Carrington et al., 1989) and ABS3 as a 9.4-kb EcoRI-Sall fragment. This plasmid is referred to as p1172. An Xbal site was introduced downstream of the PLC gene sequence, after the polyadenylation sites (see Fig. 1a) by site-directed mutagenesis to give p1172x. The ILT1α se-rodeome (Miller and Turner, 1981) is heterozygous for an insertion type adjacent the adjacent RFLP genes using PCR. Antibodies were used at 1 to 5 μl reaction with 4 × 10⁶ cell equivalents of trypansomes lysates were prepared by resuspending cells in SDS-PAGE sample buffer at 2 × 10⁶ cells/ml and immediately incubating at 100°C for 3 mins. Hypotonic lysates (Fig. 3α) were prepared by resuspending cells in TES buffer (20 mM TES, pH 7.5, 140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM EDTA, 1 mM PMSF, 50 μg/ml leupeptin) at 3 × 10⁶ cells/ml and then diluting to 2 × 10⁵ cells/ml with hypotonic dilution buffer (1 mM EDTA, 1 mM PMSF, 50 μg/ml leupeptin). The lysates were incubated for 5 min at 37°C. PI-PLC treatment involved incubation of hypotonic lysates with 4 U Bacillus cereus PI-PLC (Boehringer) per 1 × 10⁵ cells for 30 min at 37°C. In other experiments, as an alternative to hypotonic lysis, cells were lysed in 1% Triton X-100 in TES buffer at a concentration of 2 × 10⁶ cells/ml. The preparation of recombinant GPI-PLC will be described elsewhere. In all the experiments in this paper the GPI-PLC was added in sufficient quantities to completely hydrolyze the GPI anchor of the VSG in 5 min at 37°C.

**PLC Replacement Construct, pB**

The PLC replacement construct (see Fig. 1b) was designed to integrate a copy of the PLC gene into the tubulin gene cluster, which in T. brucei is comprised of an α and β tubulin gene pair tandemly arrayed 12 to 15 times. The coding sequence of the βten gene, including the termination codon, from pUT58 (CAYLA), was used to replace part of the coding sequence of an α tubulin gene from the initiation codon to the StuI site. In the adjacent β tubulin gene the entire coding sequence was precisely replaced with the PLC-gene coding sequence. The encoded mRNA therefore comprised of an α and β tubulin gene pair tandemly arrayed 12 to 15 times. The coding sequence of the βten gene, including the termination codon, from pUT58 (CAYLA), was used to replace part of the coding sequence of an α tubulin gene from the initiation codon to the StuI site. In the adjacent β tubulin gene the entire coding sequence was precisely replaced with the PLC-gene coding sequence. The encoded mRNA therefore comprises of an α and β tubulin gene pair tandemly arrayed 12 to 15 times.

**Nucleic Acids and Proteins**

Preparation, gel electrophoresis, and blotting of nucleic acids were performed by X-irradiation (550–600 radons). Chromatography (Lanham and Godfrey, 1970). Immunosuppressed mice were grown in mice and purified from blood by DEAE-cellulose chromatography (Lanham and Godfrey, 1970). Immunosuppressed mice were prepared by X-irradiation (550–600 radons).

**Trypanosomes**

The procyclic trypanosome line used, pro G Anvers, was derived from the endogenous ClaI–BstEII fragment, to give two constructs, p1172kxL and p1172kxS containing the long and short RFLP allele, respectively. The restriction sites were added to the chimeric antibiotic resistance genes using PCR.

**Trypanosome Extracts**

SDS lyases were prepared by resuspending cells in SDS-PAGE sample buffer at 2 × 10⁶ cells/ml and immediately incubating at 100°C for 3 mins. Hypotonic lysates (Fig. 3α) were prepared by resuspending cells in TES buffer (20 mM TES, pH 7.5, 140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM EDTA, 1 mM PMSF, 50 μg/ml leupeptin) at 3 × 10⁶ cells/ml and then diluting to 2 × 10⁵ cells/ml with hypotonic dilution buffer (1 mM EDTA, 1 mM PMSF, 50 μg/ml leupeptin). The lysates were incubated for 5 min at 37°C. PI-PLC treatment involved incubation of hypotonic lysates with 4 U Bacillus cereus PI-PLC (Boehringer) per 1 × 10⁵ cells for 30 min at 37°C. In other experiments, as an alternative to hypotonic lysis, cells were lysed in 1% Triton X-100 in TES buffer at a concentration of 2 × 10⁶ cells/ml. The preparation of recombinant GPI-PLC will be described elsewhere. In all the experiments in this paper the GPI-PLC was added in sufficient quantities to completely hydrolyze the GPI anchor of the VSG in 5 min at 37°C.

**GPI-PLC Assays**

These were performed using [3H]-myristyl VSG as substrate and counting the 3H released into an organic phase on phase separation (Bulow and Overath, 1986). 3 μg of VSG that contained 10,000 cpm 3H was used as substrate in a 200-μl reaction with 4 × 10⁶ cell equivalents of trypanosomes lysates in TES buffer containing 1% Triton X-100.

**In Vitro Differentiation from Bloodstream to Procyclic Form**

Trypanosomes were grown in immunosuppressed mice for 7 d, until the parasite population was predominantly stumpy in morphology. Differentiation was induced as described (Rolin et al., 1993); briefly, cells were inoculated at a density of 1 × 10⁶ cells/ml into modified DTM (Overath et al., 1986) with 15% heat-inactivated fetal calf serum and 3 mM citrate/cis-aconitate and incubated at 27°C in 4% CO₂ in air (Ziegelbauer et al., 1990).

**FACS® Analysis**

FACS® analysis was performed as previously described (Rolin et al., 1993). A mixture of three anti-sera (anti-Antat 1.1, 1.2, and 1.3) was sufficient to detect the VSG using FACS®. The anti-procyclin was the monoclonal antibody TPRI/247 (Cedarlane, Hornby, Ontario, Canada).

**Chronic Infections of Mice**

The different trypanosome stocks being tested were injected intraperitoneally (i.p.) into each of six matched C57BL6 mice using an innoculation of...
1 × 10⁶ trypanosomes, unless otherwise stated. Parasite densities in subsequent infections were determined using an improved Neubauer haemocytometer after dilution of tail blood into 0.85% ammonium chloride. The minimum detectable density was 4 × 10⁴ trypanosomes/ml. Infections were monitored daily for the first 24 d and then 3–4 times a week until 90–100 d after infection. Mice were removed from the study on exhibiting severe clinical symptoms such as inability to walk, hunched appearance, and labored breathing.

**Statistical Analysis**

Mann-Whitney tests were used to compare parasitemias at the first peak and survival times between groups. Probability values were adjusted for ties where appropriate. A probability value of ≤0.05 was taken to indicate a significant difference.

**Immunofluorescence**

Trypanosomes were purified from blood and fixed in suspension with 4% paraformaldehyde in PBS for 10 min. After washing with PBS, the cells were allowed to settle onto polylysine-coated multiwell slides. The remainder of the procedure followed standard protocols; the mouse serum was diluted 1:500.

**Results**

**Design of the PLC Elimination Constructs**

The approach taken to generate a GPI-PLC null mutant *T. brucei* cell line was targeted deletion of the gene by homologous recombination (Lee and Van der Ploeg, 1990; ten Asbroek et al., 1990; Eid and Sollner-Webb, 1991). There is a single PLC gene per haploid genome (Car- rington et al., 1989), and since *T. brucei* is diploid, two successive deletions were performed using the selectable marker cassettes neoR and hygR, coding for neomycin and hygromycin phosphotransferase, respectively. The targeting constructs were designed to affect removal of the entire PLC gene, including both 5' and 3' UTRs. The end points of the deletion were also chosen to minimize any interference with the expression of flanking genes, and the untranslated regions were removed to avoid any influence that they might exert on marker gene expression. The desired deletion resulted in the elimination of the entire PLC gene from before the dominant mini-exon addition site to beyond the polyadenylation sites, but left the processing sites of the flanking genes untouched. The gene targeting constructs are shown in Fig. 1a, along with a map of the genome before and after a favorable recombination event. The determination of the RNA processing sites for the PLC and flanking genes will be published elsewhere. The deletions were performed in procyclic trypanosomes that do not express GPI-PLC and thus should present no phenotype and be at no selective disadvantage. Cells were subsequently induced to differentiate to a form that usually expresses the enzyme.

**Elimination of the PLC Gene**

Before electroporation, constructs were linearized to increase the efficiency of stable integration and to favor a gene replacement (ten Asbroek et al., 1993). Trypano-
correspond to the HindIII fragments of DNA. Washed in 0.1 M NaCl, 0.05 M Tris-HCl (pH 8) with probe B or probe PLC, Southern blot of BamHI-digested genomic DNA probed with 5′-biotinylated PLC1 coding sequence probe to the PLC1-null clone were digested with BamHI and probed with probe B or PLC1-coding sequence as indicated. Blots were washed in 0.1× SSC, 0.1% SDS for 2 h at 60°C. Sizes on the left correspond to the HindIII fragments of λ DNA.

Figure 2. Southern analysis demonstrating elimination and subsequent replacement of the PLC gene in EATRO 1125 proG procyclic T. brucei. About 4 μg of genomic DNA from (a) parent EATRO 1125 proG cells, (b) a G418-resistant line cloned after a single PLC knock-out, (c) the PLC null mutant (PLC−) clone and (d) a PLC− null clone were digested with BamHI and probed with probe B or PLC-coding sequence as indicated. Blots were washed in 0.1× SSC, 0.1% SDS for 2 h at 60°C. Sizes on the left correspond to the HindIII fragments of λ DNA.

Confirmation of Phenotype and Lack of Redundancy
Since PLC− procyclic trypanosomes were able to differentiate to bloodstream forms it is evident that GPI-PLC activity is not necessary for these developmental steps. To confirm the absence of GPI-PLC activity, the cleavage of mfVSG to sVSG by endogenous enzymes was assayed in PLC− and control cells. A Western blot was used to determine whether the CRD was exposed (Fig. 3 a). The assay would also detect any alternative activity, such as another phospholipase C or D, capable of hydrolyzing the GPI anchor to expose the CRD.

On SDS lysis there is very rapid inactivation of GPI-PLC, and the reaction of the VSG with the anti-CRD is weak or nonexistent. Recognition of some mfVSGs has been observed previously and seems to depend on an epitope involving the galactose branch and ethanolamine phosphate bridge of the GPI anchor (Zamze et al., 1988). The varying degree of galactosylation in different VSG anchors may explain the differing reactivity of their intact form with anti-CRD antibodies. The difference in mfVSG electrophoretic mobility between the control and null cells (Fig. 3 a, compare lanes 1 and 4) suggests that the predominant VSG in the two populations is different. This is consistent with the slight recognition of the mfVSG in the SDS lysate of the PLC−, but not control trypanosomes.

On hypotonic lysis and incubation at 37°C, the VSG in the control trypanosomes but not PLC− trypanosomes became strongly CRD positive. This confirms the absence of GPI-PLC activity in the null mutant and, furthermore, indicates that there is no alternative endogenous metabolic route to convert mfVSG to sVSG. The VSG in the PLC− cells became CRD positive on incubation with exogenous B. cereus PI-PLC. This result indicates that the GPI anchor on the VSG in the PLC− cells contains PI and the core oligosaccharide recognized by anti-CRD antibodies, suggesting that the anchor structure is normal.

Absence of GPI-PLC activity in the PLC− trypanosomes was confirmed by assaying detergent lysates for their ability to release tritium from [3H]-myristyl VSG. Activity in the wild-type cells was 45 pmol/min/mg protein, which is similar to previously reported values (Bülow and Overath, 1986) and in null mutant cells was <0.5 pmol/min/mg protein, the limit of reliable detection of the activity. It is not clear whether the removal of a single myristic acid from the GPI anchor by a phospholipase A would result in either VSG shedding or exposure of the CRD epitope. However, failure to release tritium into the organic phase during the standard assay argues against the presence of such a phospholipase in the PLC− cells.

A further experiment was performed to investigate if any other modifications to the GPI anchor could be detected after lysis. In this case Triton X-100 lysis was used and the lysate incubated at 37°C for 20 or 40 min with or without added recombinant GPI-PLC. In the absence of added GPI-PLC, no anti-CRD binding was detected either at 20 or 40 min, similar to the previous result. Additionally, if the lysate was incubated first for 20 min in the absence of added GPI-PLC and the enzyme then was added for the second 20 min of the incubation, the same strength of anti-CRD signal was obtained (Fig. 3 b). This result means that, in the absence of GPI-PLC gene, no modifications occurred to the GPI anchor that would affect hydrolysis by GPI-PLC, such as acylation, hydrolysis by glucosidases, or hydrolysis by a phospholipase D.

It has already been reported that, unlike wild-type, PLC− trypanosomes do not release sVSG in stress conditions (Rolin et al., 1996).

Differentiation of PLC− Trypanosomes from Bloodstream to Procyclic Form
On ingestion by tsetse flies, bloodstream trypanosomes differentiate to procyclic forms, a process that can be mim-
Figure 3. Analysis of the VSG in PLC\(^-\) and control bloodstream trypanosomes. (a) Cell extracts were prepared by SDS lysis, hypotonic lysis, and with addition of exogenous B. cereus PI-PLC to the hypotonic lysate. Control cell extracts were loaded in lanes 1–3, and the equivalent PLC\(^-\) trypanosome extracts in lanes 4–6. The top panel shows a Coomassie blue-stained gel (2 \(\times\) 10\(^5\) cells/track). Beneath is an equivalent gel (2 \(\times\) 10\(^5\) cells/track) that has been Western blotted and probed with an anti-CRD polyclonal antibody. (b) Trypanosome extracts were prepared by triton lysis and incubated at 37°C in the absence or presence of GPI-PLC. The hydrolysis of the VSG by the GPI-PLC was monitored by Western blotting the whole reaction using anti-CRD.

Difficulties in vitro (Ziegelbauer et al., 1990). Since the PLC\(^-\) mutant was first generated as a procyclic cell line, this differentiation would complete the life cycle. Bloodstream-form cells were harvested from irradiated mice 7 d after infection when the population was predominantly stumpy and hence predisposed to synchronous differentiation (Ziegelbauer et al., 1990). A plot of cell density against time after cells were induced to differentiate is given in Fig. 4. The control trypanosomes displayed a 15-h lag before exponential growth of procyclic forms commenced. This is similar to results previously reported with stumpy-form AnTat 1.1 cells (Ziegelbauer et al., 1990; Rolin et al., 1993). The lag and subsequent growth rate of the PLC\(^-\) trypanosomes were indistinguishable from the control and are within the normal range expected for this cell line. The distinct morphological changes that occur in wild-type cells during this time (Rolin et al., 1993) were also apparent during differentiation of the PLC\(^-\) cells (data not shown).

FACS\(^\text{®}\) analysis (Roditi et al., 1989; Ziegelbauer et al., 1990) was used to follow the kinetics of appearance of cell surface procyclin and loss of VSG in the PLC\(^-\) trypanosomes. In preliminary experiments, the VSG expressed by the nonclonal PLC\(^-\) trypanosomes was investigated by immunofluorescence. Of 12 antibodies tried (anti-AnTat 1.1 to anti-AnTat 1.12), 4 (AnTat 1.1, 1.2, 1.3, and 1.6) were found to react weakly with some cells in the population. The strength of the fluorescence indicated that the VSG(s) expressed by the PLC\(^-\) trypanosomes was slightly cross-reactive with these antibodies as opposed to actually being one of these VSGs. A mixture of three of the antisera (AnTat 1.1, 1.2, and 1.3) was sufficient to produce a signal for the VSG that was detectable on FACS\(^\text{®}\) analysis. Fig. 5 shows that surface VSG has been completely replaced by procyclin between 7 and 9 h after initiation of differentiation. Once again, this is consistent with previous studies on this cell line (Rolin et al., 1993). Furthermore, the transient activation of adenylate cyclase, which follows VSG release during differentiation, occurs normally in the PLC\(^-\) trypanosomes (Rolin et al., 1996). Finally, when bloodstream forms were fed to tsetse flies, PLC\(^-\) trypanosomes established a typical procyclic infection in the midgut (data not shown). This indicates that the essentially normal differentiation observed in vitro also occurs in vivo.

**PLC\(^-\) Trypanosomes Can Establish a Persistent Infection**

The initial stablates of the bloodstream form of PLC\(^-\) trypanosomes were produced in immunosuppressed mice and so had not interacted with the host immune system. To establish a persistent infection in an immunologically competent mouse, trypanosomes have to exchange the metacyclic VSG for a bloodstream form VSG, and this occurs in the majority of the population (Esser and Schoenbecher, 1985). Subsequently a small subset of the population undergoes antigenic variation to avoid antibody-mediated lysis. To test whether the VSG-shedding properties of GPI-PLC play an essential part in these two processes, infections were established in laboratory mice. Persistent infections lasting \(>50\) d were readily observed with the PLC\(^-\) bloodstream-form population. Parasitemic profiles for six individual mice for the first \(50\) d after infection are given in Fig. 6. Each mouse shows the same basic pattern that is in turn broadly similar to the profiles observed previously for six cloned T. brucei stocks (Turner et al., 1995). An initial peak of parasitemia is followed by a relapse and recrudescence (‘trough’) to a comparatively level plateau, punctuated by one or more further troughs.

Although it is barely credible that such an infection could persist without antigenic variation, two experiments were performed to show that VSG switching occurs in the PLC\(^-\) trypanosomes. A clonal line of PLC\(^-\) trypanosomes was first established by infecting immunosuppressed mice...
with single trypanosomes, and stabilates were made 10–14 d later. Three mice were then infected using a single stabili-
tate. The first mouse was used to prepare trypanosomes for immunofluorescence during the first peak of para-
sitemia. The second mouse was used to prepare serum just after the first peak of parasitemia had declined, and the third mouse was used to prepare trypanosomes for immu-
nofluorescence during the second peak of parasitemia. The results are shown in Fig. 7; the serum clearly recog-
nizes trypanosomes in the first peak, but not in the second.

In the second experiment, trypanosomes from the first and a subsequent peak were used to produce detergent lys-
sates. The triton lysates were then treated with recombi-
nant GPI-PLC before Western blotting with anti-CRD (Fig. 7 b). Both populations are expressing readily detect-
able VSG(s) that have different profiles on densitometric analysis.

**PLC** Trypanosomes Have an Altered Parasitemic Profile when Compared with Control Cells in Model Infections

The course of chronic infection of mice with control try-
panosomes was monitored in parallel with the PLC**

infections described above. Passage through a tsetse fly can
greatly alter the growth characteristics of a trypanosome cell line (Turner, 1990). Therefore, the most comparable trypanosome population available was used as a control in infections. This population had been derived from the same procyclic cell line as the PLC mutant and was transmitted through tsetse flies in parallel, but had a chloramphenicol acetyl transferase-hygromycinB gene construct integrated into the tubulin gene cluster (Berberof et al., 1995). Since isolation from tsetse flies, both of the try-
panosome stocks had been amplified twice in immunosup-
pressed NMRI mice and stabilates prepared from the second mouse. These stabilates were used to infect immu-

Figure 5. FACS® analysis showing the loss of VSG and the appearance of procyclin during the in vitro differentiation of PLC**

trypanosomes to procyclic forms.

Figure 6. The course of infection with PLC**

trypanosomes in six individual mice. Parasite densities are expressed as log$_{10}$ number of trypanosomes/ml of blood.
tions with PLC\(^{-}\) trypanosomes. These were designed to investigate whether prolonged growth in mice and inoculum size altered the parasitemia. Syringe passaging of laboratory trypanosome lines through mice is a standard method for artificially increasing the level of parasitemia achieved during infection. Thus two sets of six mice were infected with PLC\(^{-}\) trypanosomes that had undergone 22 additional syringe passages in immunosuppressed CFLP mice. One set of mice was infected with \(1 \times 10^6\) trypanosomes as above and a second set with \(1 \times 10^7\) trypanosomes.

The PLC\(^{-}\) trypanosomes show a more than threefold decrease in first peak parasite density that was significantly different (\(P = 0.01\)) compared to control cells. In this experiment, PLC\(^{-}\) trypanosome density never reached \(1 \times 10^8\) cells/ml during the first 20 d of infection and for most of the time the levels were \(<1 \times 10^7\) cells/ml. The control cells showed a higher overall parasitemia, \(>1 \times 10^8\) cells/ml for an average of 17.5% and \(<1 \times 10^7\) cells/ml for 32.5% of the first 20 d. Repeatedly passaging the PLC\(^{-}\) trypanosomes resulted in an increased parasitemia in the first 20 d, although all parameters indicated that the parasitemia was still lower than with the control trypanosomes and still statistically significant in the case of the lower inoculum dose (\(P = 0.01\)).

The survival time can be taken as an indication of the virulence of an infection. The mean survival time of mice infected with the control trypanosomes was 28 d, and was

Figure 7. Antigenic variation in PLC\(^{-}\) trypanosomes. (a) Immunofluorescence of trypanosomes from the first and a successive peak of parasitemia using antiserum from a mouse that had just cleared the first peak. (b) A Western blot, probed with anti-CRD, showing the effect of GPI-PLC treatment of total cell lysates from PLC\(^{-}\) trypanosomes from successive peaks of parasitemia. The enlarged panel shows the CRD-positive VSGs from the two peaks; the densitometric scan of this region is shown below. Bar, 50 \(\mu\)m.

Figure 8. The course of infection in mice infected with control and PLC\(^{-}\) trypanosomes for comparison of parasitemias. Results are shown as geometric mean \(\pm 2\) standard errors, \(n = 6\). Arrowheads indicate the survival time of individual mice.
significantly ($P = 0.01$) lower than in $PLC^−$ trypanosome infections, where the mean survival time was 81 d, and three of the six mice were still alive at the end of the experiment on day 99. Passaging and inoculum size of the $PLC^−$ trypanosomes had no significant effect on the survival time of the infected mice despite the higher overall level of parasitemia compared with the unpassaged $PLC^−$ trypanosomes. Survival time remained significantly different from control infections at both inoculation size ($P = 0.05$ and 0.01 for $10^6$ and $10^7$ trypanosomes, respectively).

**The Altered Parasitemia in $PLC^−$ Trypanosome Infections is Partially Rescued by Low Level GPI-PLC Expression**

The results above clearly showed that mice infected with $PLC^−$ trypanosomes had an altered parasitemic profile when compared to the best available control. To address the question as to whether this alteration was due to the absence of the $PLC$ gene, or some other event, a copy of the $PLC$ gene was returned to the $PLC^−$ trypanosome. It was decided to place the $PLC$ gene in the tubulin locus (to produce $PLC^+$ trypanosomes) as opposed to returning it to the endogenous position, as the latter manipulation could conceivably repair any lesion in a flanking gene. The targeting construct employed ble$^R$ as a selectable marker and is shown in Fig. 1. Southern analysis of BamHI digested DNA from one phleomycin resistant clone is shown in Fig. 2 and shows that the $PLC$ probe hybridises to a 6.6-kb band, as expected after the desired integration into the tubulin locus.

Phleomycin-resistant clones were transmitted through tsetse flies, and the expression of GPI-PLC characterized by Western blotting and assaying mVSG to sVSG conversion on hypotonic lysis. The $PLC^+$ trypanosomes were found to contain GPI-PLC protein but, by comparison with a twofold dilution series of AnTat 1.1 cells (Fig. 9), the level of GPI-PLC in $2 \times 10^6$ $PLC^+$ trypanosomes was roughly equivalent to that in only $1.25 \times 10^5$ AnTat1.1 cells. Thus the $PLC^+$ trypanosomes contain approximately one sixteenth of the wild-type, bloodstream-form GPI-PLC level. The presence of GPI-PLC activity was tested in two assays. The first was detergent lysis of $PLC^+$ trypanosomes; in this assay the endogenous VSG became anti-

**Table I. Comparison of Parasitaemias Produced on Infection of Mice with Various Trypanosome Lines; Mann-Whitney Tests Were Used to Compare Experimental Groups with the Control (Ia) or PLC− with PLC+ (Ib), and p Values Are Given in Brackets**

| Trypanosome line | Median no. of troughs* | Parasitaemia at first peak | No. of days pd $> 1 \times 10^3 (\% )$ | No. of days pd $< 1 \times 10^2 (\% )$ | Mean survival time |
|------------------|------------------------|-----------------------------|-----------------------------------------|---------------------------------------|------------------|
| Control          | 1                      | $1.4 \times 10^4$           | 3.5 (17.5)                              | 6.5 (32.5)                            | 28.0             |
| $PLC^− 22p$      | 3                      | $4.1 \times 10^3 (P = 0.01)$| 0                                       | 12.8 (64.2)                           | 81.3 ($P = 0.01$) |
| $PLC^− 22p 1 \times 10^7$ | 1–2                  | $7.4 \times 10^3 (P = 0.01)$| 1.2 (6.0)                              | 11.2 (56)                             | 72.8 ($P = 0.05$) |
| $PLC^+$          | 1–2                    | $9.4 \times 10^7 (P = 0.01)$| 2.2 (10.8)                             | 9.7 (48.3)                            | 56.7 ($P = 0.26$) |

a The mean number of days on which parasite density ($pd$) was measured over 93 d. The data are summarized in Table I b. The parasitemia and mouse survival data for the $PLC^−$ parasites in the two experiments were slightly different as might be expected, but statistical comparison of parasitemias at first peak and survivorship showed no significant differences

$^{*}$ An trough is defined as a drop in parasitemia greater than one order of magnitude between adjacent time points (calculated for the first 40 d).

$^{\dagger}$ Geometric means of parasite density at the peak of the first wave of parasitaemia ($n = 6$, except for $PLC^− 22p$, where $n = 5$).

$^{\ddagger}$ The mean number of days on which parasitemic density ($pd$) was $> 1 \times 10^6$ cells per ml of blood, or $< 1 \times 10^5$ cells per ml of blood. These were determined for the first 20 d after infection and are expressed as percentages in parentheses.

**Figure 9.** Expression of GPI-PLC protein in $PLC^+$ trypanosomes. Lysates of bloodstream trypanosomes from three lines; AnTat 1.1, $PLC^−$, and $PLC^+$ trypanosomes were Western blotted and probed with anti-GPI-PLC. All samples are from the same blot and were probed simultaneously.
Parasite density compared to the expression coincides with an increase in the parasitemia. PLC
panosomes are capable of completing an entire life cycle. Prepared by SDS or Triton lysis, Western blotted, and probed with anti-CRD. All samples are from the same blot and were probed simultaneously.

\( P = 0.30 \) and \( P = 0.47 \), respectively. Low level GPI-PLC expression coincides with an increase in the parasitemia over the first 20 d, with a significant increase in first peak parasite density compared to the \( PLC^- \) infections (\( P = 0.01 \)). In addition there is a downward trend in mean survival time to values intermediate between the \( PLC^- \) and wild-type trypanosomes, although this is not statistically significant.

**Discussion**

The function of the GPI-PLC in \( T. brucei \) has been the cause of much speculation, as the enzyme has an activity that could theoretically facilitate the rapid shedding of the protective VSG coat. A GPI-PLC null mutant has been made by targeted gene deletion to test whether the ability of the GPI-PLC to hydrolyze the GPI anchor of the VSG, which is observed on both lysis and stress, is essential in vivo. The generation of the mutant was facilitated by the preliminary analysis of the GPI biosynthetic pathway in trypanosomes. This in turn suggests that GPI-PLC is indeed responsible for the low rate of CRD-positive VSG shedding that occurs from living trypanosomes (Bülow et al., 1989; Seyfang et al., 1990), and this could be confirmed if it becomes possible to establish \( PLC^- \) trypanosomes in culture. The function of this low rate of VSG shedding remains unclear. The viability of the GPI-PLC null mutant indicates that it is not an essential process in VSG turnover, although it may well affect its kinetics. If the steady turnover of surface VSG in wild-type cells (Seyfang et al., 1990) depends in part on the action of GPI-PLC, coat exchange in the \( PLC^- \) trypanosomes might be expected to take longer and involve a longer period of double VSG expression. The possible importance of this in determination of parasitemic profile is highlighted by a certain mathematical model of antigenic variation (Agur et al., 1989). Although a decrease in efficiency of coat exchange may contribute to the change in overall infection dynamics exhibited by the mutant \( PLC^- \), it cannot explain certain characteristics such as the reduced height of first parasitemic peak.

In addition to the observed hydrolysis of the VSG anchor, it has been suggested that GPI-PLC may play a role in GPI metabolism or that its primary function may be in PI hydrolysis. The experiments in this paper allow some observations to be made although they were not primarily designed to address this subject.

Cleavage of the VSG from \( PLC^- \) trypanosomes by bacterial PI-PLC producing CRD-positive VSG strongly suggests that the GPI anchor structure is normal and argues against a role for GPI-PLC in GPI biosynthesis. Also, preliminary analysis of the GPI biosynthetic pathway in \( PLC^- \) trypanosomes indicates that the normal intermediates are present (Ferguson, M., and M.-L. Güther, personal communication).

However, GPI-PLC may yet play a part in the kinetics of the biosynthetic pathway, perhaps in the catabolism of excess GPI precursors since flux through the pathway is apparently 10-fold in excess of absolute protein anchoring requirements (Masterson and Ferguson, 1991). Such a catabolic role may involve cleavage of one or more of the cytoplasmically disposed GPI precursors in the cytoplasmic leaflet of the ER and would have to be tightly controlled to preserve sufficient GPI to form VSG anchors. When GPI-PLC is expressed in an heterologous system (Leishmania major) any such regulation is absent and cells secrete gp63 in a CRD-negative form because it never receives a GPI anchor (Mensa-Wilmot et al., 1994). It is not clear to what extent these results are applicable to \( T. brucei \).
In *T. brucei*, there is a dynamic equilibrium between the GPI-PLC-sensitive end-product of the biosynthetic pathway that is attached to the VSG (glycolipid A) and an acylated, GPI-PLC-resistant, form (glycolipid C; Güther et al., 1994; Güther and Ferguson, 1995). Glycolipid C is not an obligatory GPI precursor (Masterson and Ferguson, 1991) and, unlike most GPI intermediates, is predominantly localized in the luminal leaflet of the ER (Vidigalirêne and Menon, 1994). The fact that it is kept both chemically and topologically secure from GPI-PLC attack is intriguing. The role of glycolipid C is unclear, although it may act as a store of GPI anchors and hence as a buffer against fluctuating demands for GPI (Güther et al., 1994). Preliminary analysis of the GPI biosynthetic pathway in *PLC* trypanosomes indicates that there is no gross alteration in the glycolipid A to C ratio, or accumulation of excess GPI (Ferguson, M., and M.-L. Güther, personal communication). Although we cannot rule out a role for GPI-PLC in catabolism of GPIs in vivo, it is clear that such a role is not essential.

Any potential role of the GPI-PLC in PI metabolism is more difficult to discuss in the absence of much knowledge about signal transduction in trypanosomes. It is not possible, at this stage, to rule out a non-essential role for the GPI-PLC involving PI hydrolysis. The loss of such a function in *PLC* trypanosomes may be rescued by a redundant activity that does not metabolise GPI.

The second finding is that *PLC* trypanosomes have an altered parasitemia in laboratory mice. The main differences between the *PLC* and control trypanosomes are the degree of parasitemia in the early stages of a chronic infection and the survival time of infected mice. However, before an attenuated phenotype can be firmly assigned to *PLC* trypanosomes it should be confirmed in independently derived mutants, which are not yet available. Data presented in this paper are based on the behavior of one clone only. It should also be noted that the natural hosts for *T. brucei* are mammals in sub-Saharan Africa and not laboratory mice; the course of infection with *PLC* trypanosomes in indigenous cattle may provide a more clearly defined phenotype. In addition, confirmation should come from a full phenotype rescue, which may only be possible if *PLC* trypanosomes can be modified to express GPI-PLC at wild-type levels. The possible partial rescue observed in *PLC* trypanosomes is however encouraging.

There is variability in the parasitemia caused by different isolates of *T. brucei*, but any one isolate produces a consistent course of infection (Turner et al., 1995). The progress of the initial stages of parasitemia (first 20 d) observed in both control and *PLC* trypanosomes are within the range reported for these different trypanosome isolates (Turner et al., 1995). However, compared to the control trypanosomes, the initial stages of parasitemia are reduced in *PLC* trypanosomes, an effect that is partially reversed in *PLC* trypanosomes. How could the absence of GPI-PLC affect the initial parasitemia? Either the growth rate of the *PLC* trypanosomes is reduced by an elongation of the cell cycle time and/or increased rate of differentiation to the nondividing stumpy forms. Alternatively the *PLC* trypanosomes could be killed more efficiently by the host immune system. One possible mechanism for the latter is that on immune lysis wild-type trypanosomes release sVSG into the blood of the mouse which then acts as a decoy to anti-VSG immunoglobulins as they are produced. It is worth noting that blood with $1 \times 10^8$ trypanosomes/ml contains roughly 100 $\mu g$/ml of VSG.

The second clear difference between control and *PLC* trypanosomes is the survival time of the infected mouse, which was unaffected by passage number and inoculum size. Although survival time showed a downward trend in *PLC* infections compared to *PLC* infections, the change was not statistically significant. It remains to be seen whether *PLC* trypanosomes can be modified to express wild-type levels of GPI-PLC and, if so, whether this would result in a complete return to wild-type virulence. The survival time of *PLC* infected mice was apparently longer than those previously observed for a range of isolates (Turner et al., 1995), although these values were obtained in an inbred mouse strain (BALB/c as opposed to CFLP in this study). The survival time, which can be used as a measure of virulence, correlates with the initial parasitemia (Turner et al., 1995), and will be influenced by the genotype and epigenetic state of the trypanosome and the host immune system (Barry and Turner, 1991). Using the data available, it is not possible to provide a simple explanation of how the absence of GPI-PLC activity might reduce the virulence of trypanosomes. However, it is known that *T. brucei* causes immunosuppression in the host (Askonas, 1985) and this must be a component of virulence. If GPI-PLC activity is necessary for the immunosuppression then it might be expected that *PLC* trypanosomes would be less virulent than the equivalent wild-type strain.

We would like to thank Peter Overath for encouragement and his helpful comments on the manuscript, Mike Turner for his help with the manuscript and the statistics, and Lucia Cardoso de Almeida for discussions and encouragement.

This work was funded by the Medical Research Council and the Wellcome Trust.

Received for publication 8 November 1996 and in revised form 17 July 1997.

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