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.

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 NH_2-terminal domain comprising ~350 residues and a COOH-terminal domain(s) of 50 to 100 residues (Johnson and Cross, 1979). The structure of the NH_2-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 (mfVSG) 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 mfVSG 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

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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.

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 K_m 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 1.13×10^7 molecules of the VSG (Jackson et al., 1985) and $\sim 2.7 \times 10^7$ molecules of PI (Voorheis and Martin, 1980; Carroll and McCrorie, 1986). However, the observation that the PLC is active against 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 $\sim 3 \times 10^4$ molecules of GPI-PLC/trypanosome (Hereld et al., 1986) and at an estimated turnover rate of 100 to 700 mfVSG 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., 1989*b*). 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 mfVSG 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 mfVSG 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., 1989*a*). 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 mfVSG.

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., 1989*b*) 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 protease (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^R and hyg^R antibiotic resistance genes were constructed to a standard format. The procyclin mini-exon acceptor site and 5′ 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 λBS2 (Carrington et al., 1989) and λBS3 as a 9.4-kb EcoRI–SalI fragment. This plasmid is referred to as p1172. An XbaI site was introduced downstream of the GPI-PLC gene, after the polyadenylation sites (see Fig. 1 *a*) by site-directed mutagenesis to give p1172x. The ILTar 1 serodeme (Miller and Turner, 1981) is heterozygous for an insertion type RFLP in the intergenic region upstream of the GPI-PLC gene (see Fig. 1 *a*). Since the constructs were originally designed to target this trypanosome line, both alleles were cloned as PCR products, and a KpnI site was introduced into each by site directed mutagenesis (see Fig. 1 *a*). These were subcloned back into p1172x as a ClaI–BstEII fragment, in place of the endogenous ClaI–BstEII fragment, to give two constructs, p1172kxL and p1172kxS containing the long and short RFLP allele, respectively. The trypanosome line actually used for the gene deletion (see below) is homozygous for the long RFLP.

The entire GPI-PLC gene was then removed from these plasmids as a KpnI–XbaI fragment and replaced with the neo^R expression cassette (p1172kxL) or the hyg^R cassette (p1172kxS) to give pLN and pSH, respectively. The restriction sites were added to the chimeric antibiotic resistance genes using PCR.

PLC Replacement Construct, pB

The *PLC* replacement construct (see Fig. 1 *b*) 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 ble^R 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 GPI-PLC coding sequence. The encoded mRNA therefore consists of the GPI-PLC coding sequence with β tubulin 5′ and 3′ UTRs. Where necessary, restriction enzyme sites were added using PCR.

Trypanosomes

The procyclic trypanosome line used, pro G Anvers, was derived from the EATRO 1125 (Van Meirvenne et al., 1975) stock by recent passage through tsetse flies and kept in culture for only 40 d after isolation from the fly. Procyclics were cultured in SDM-79 (Brun and Schönenberger, 1979) with 10% vol/vol heat-inactivated fetal bovine serum. Bloodstream forms 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).

Nucleic Acids and Proteins

Preparation, gel electrophoresis, and blotting of nucleic acids were performed as described previously (Carrington et al., 1987). SDS-PAGE and Western blotting were performed using standard techniques. Primary antibodies were used at 1 to 5 μg/ml. The secondary antibodies were peroxidase-conjugated donkey anti–rabbit immunoglobulin or peroxidase-conjugated rabbit anti–mouse immunoglobulin and were used at the manufacturer's recommended concentrations (Jackson ImmunoResearch Laboratories, West Grove, PA). Detection was by chemiluminescence (Amersham, Intl., Arlington Heights, IL). Alternatively, secondary antibody was alkaline phosphatase-linked anti–rabbit immunoglobulin (Boehringer Mannheim, Indianapolis, IN), and the detection used a chromogenic substrate.

Antibodies

Anti–GPI-PLC was prepared by immunizing a rabbit with a λcro-β-galactosidase–GPI-PLC fusion protein. This was prepared by cloning the end-filled DraI–EcoRI fragment from pBS1, containing a GPI-PLC cDNA (Carrington et al., 1989), into the SmaI site of pEX3 (Genofit, Grand-Lancy, France). The fusion protein was insoluble and was separated from the other insoluble proteins by preparative SDS-PAGE. The antibodies were affinity purified on a glutathione-S-transferase–GPI-PLC fusion protein (gst-plc) attached to Sepharose beads. The gst-plc was made by cloning the entire GPI-PLC coding sequence into the EcoRI and BamHI sites of pGEX2T (Pharmacia Fine Chemicals, Piscataway, NJ), the restriction sites being added to the cDNA using PCR. The gst-plc was insoluble and was purified by preparative SDS-PAGE. The anti-CRD was a kind gift of Dr. Paul Englund (Johns Hopkins Medical School, Baltimore, MD; see Fig. 3 *a*) and Dr. Linda Allen (Cambridge University, Cambridge, UK; see other figures).

Electroporation and Transformation of Procyclic Form T. brucei

Procyclic trypanosomes were grown to a density of 6 to 8×10^6 cells/ml, washed, and resuspended in ZPFM solution (Zimmerman, 1982) at a density of 4×10^7 cells/ml. 300-μl aliquots were mixed with 15 μg DNA in 4-mm cuvettes and electroporated twice at 1.5 kV, 25 μF, 200 ohms in a gene pulser (Bio Rad, Hercules, CA). These cells were used to seed a 10-ml culture, and antibiotic selection was introduced 24 h after electroporation. For elimination of the *PLC* gene, cells were first transformed to G418 resistance, using pLN, and then the uncloned population transformed to hygromycin resistance, using pSH. Doubly resistant trypanosomes were cloned by dilution into 40% vol/vol fresh conditioned medium with antibiotic selection. A single PLC^- clone was converted to bloodstream form by transmission through tsetse flies. For *PLC* replacement, the same procyclic PLC^- was transformed to phleomycin resistance using pB and cloned as above before tsetse transmission.

Trypanosome Extracts

SDS lysates were prepared by resuspending cells in SDS-PAGE sample buffer at 2×10^8 cells/ml and immediately incubating at 100°C for 3 mins. Hypotonic lysates (Fig. 3 *a*) 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 3×10^9 cells/ml and then diluting to 2×10^8 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 1×10^6 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 2×10^8 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 [^{3}H]-myristyl VSG as substrate and counting the ^{3}H released into an organic phase on phase separation (Bülow and Overath, 1986). 3 μg of VSG that contained 10,000 cpm ^{3}H was used as substrate in a 200-μl reaction with 4×10^6 cell equivalents of trypanosomes lysed 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 1×10^6 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% CO_2 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 CFLP mice using an innoculation of

1×10^6 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 4×10^4 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 (Carrington et al., 1989), and since *T. brucei* is diploid, two successive deletions were performed using the selectable marker cassettes neo^R and hyg^R, 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. 1 *a*, 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-

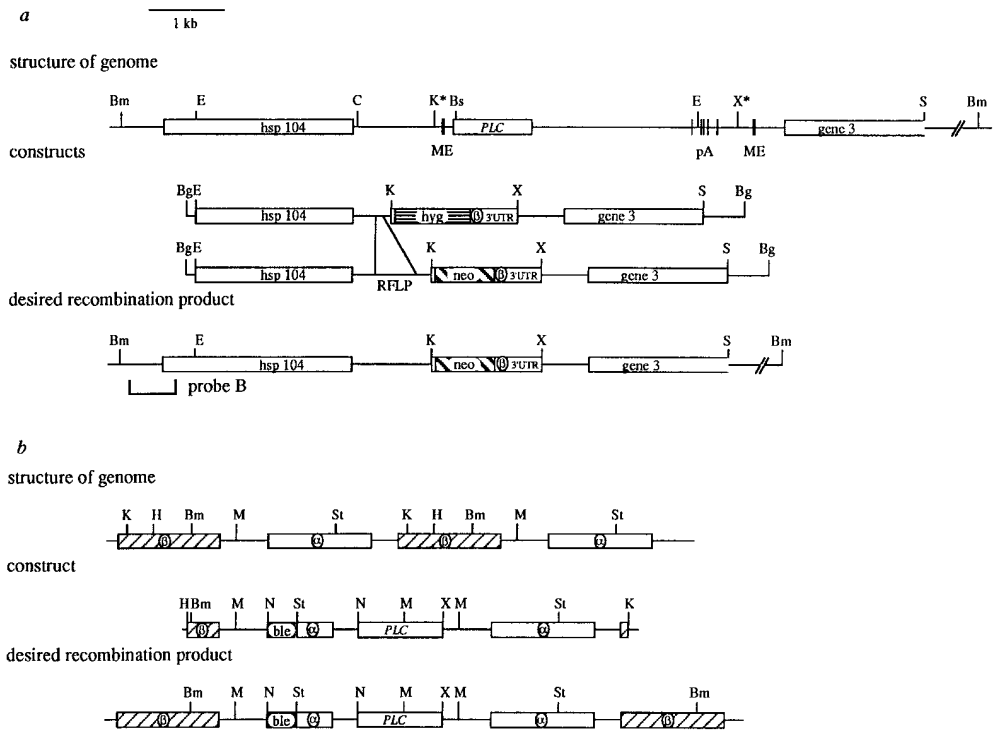

Figure 1. Structure and restriction enzyme maps of the *PLC* and tubulin loci, the constructs with which they were targeted, and the desired homologous recombination products. None of the constructs contains promoters but rely on polycistronic transcription for selectable marker expression. (*a*) Deletion of the *PLC* gene; structure of genome shows the sites of polyadenylation (pA) for the *PLC* gene and the main sites of mini-exon addition (*ME*) for both the *PLC* gene and the downstream gene, gene 3. These were mapped by RNase protection analysis (data not shown). Boxes represent coding sequences. *K** (KpnI) and *X** (XbaI) mark the positions of restriction sites introduced into genomic clones by site-directed mutagenesis. Constructs shows the *PLC* elimination constructs in which the *PLC* gene has been replaced by a chimeric expression cassette. The two constructs differ in the presence of either the long or short version of an RFLP in the intergenic region. The BglI (*Bg*) sites were used to linearize constructs before electroporation. Desired recombination product shows the genome after targeting. (*b*) *PLC* replacement. Structure of genome shows a representative section of the *T. brucei* tubulin gene cluster. Construct shows the *PLC* replacement construct, based on a PstI to PstI fragment that includes two complete α tubulin genes separated by one entire β tubulin gene. BamHI sites were used in Southern analysis of transformed cell lines. The other restriction sites were involved in construction: BstEII (*Bs*), ClaI (*C*), HindIII (*H*), MluI (*M*), NcoI (*N*), StuI (*St*).

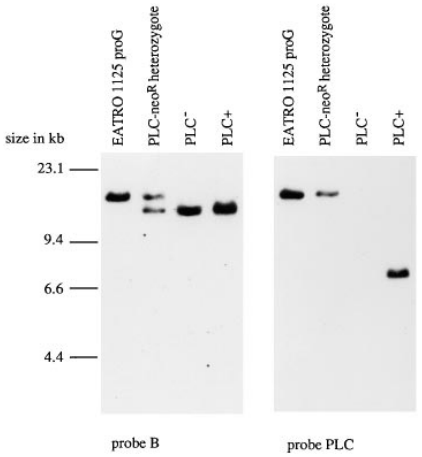

Figure 2. Southern analysis demonstrating elimination and subsequent replacement of the *PLC* gene in EATRO 1125 proG procyclic *T. brucei.* About 4 μg of genomic DNA from (*a*) parent EATRO 1125 proG cells, (*b*) a G418 resistant line cloned after a single *PLC* knock-out, (*c*) the *PLC* null mutant (*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.

somes were first transformed to G418 resistance and the uncloned population subsequently transformed to hygromycin resistance before clonal cell lines were established. Southern analysis of doubly resistant clones showed that the desired recombinational event occurred with high efficiency (14/16 events analyzed, data not shown). A Southern blot of BamHI-digested genomic DNA probed with 5′ flanking DNA (Fig. 1 *a*, *probe B*) is a way to distinguish between construct insertion and gene replacement. Such an analysis of the *PLC*$^-$ clone selected for continued study is shown in Fig. 2, in comparison with wild-type EATRO 1125 proG and a heterozygous G418-resistant clone displaying a single *PLC* gene deletion. There is no hybridization of a *PLC* coding sequence probe to the *PLC*$^-$ genomic DNA (Fig. 2). Absence of the *PLC* gene was also confirmed by PCR analysis (data not shown).

As expected, the *PLC*$^-$ procyclic trypanosomes showed no gross alterations in morphology or growth rate. A single *PLC*$^-$ clone was transmitted through tsetse flies. When trypanosomes were visible in saliva from a fly they were used to infect an immunosuppressed mouse. After further amplification in an irradiated mouse, stabilates were prepared.

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 a 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 [^{3}H]-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 gluconases, 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-

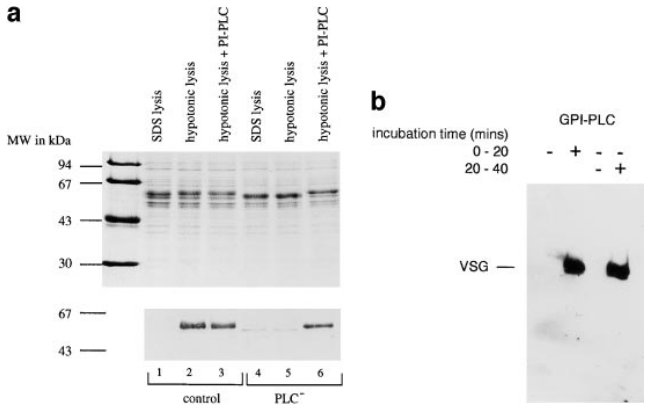

Figure 3. Analysis of the VSG in *PLC*$^-$ and control bloodstream trypanosomes. (*a*) Cell extracts were prepared by SDS lysis, hypotonic lysis, and with addition of exogenous *B. cereus* PI-PLC to the hypotonic lysate. Control cell extracts were loaded in lanes *1–3*, and the equivalent *PLC*$^-$ trypanosome extracts in lanes *4–6.* The top panel shows a Coomassie blue-stained gel (2×10^6 cells/track). Beneath is an equivalent gel (2×10^5 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.

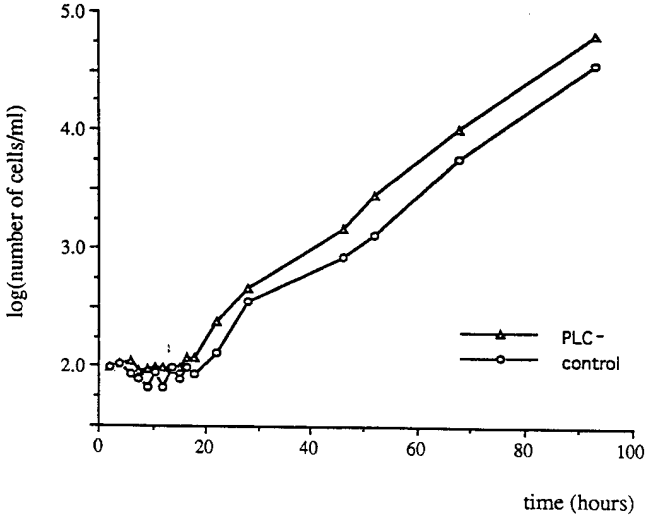

Figure 4. Time course of trypanosome growth on differentiation of control and *PLC*$^-$ trypanosomes from bloodstream to procyclic forms in vitro.

icked 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 in morphology 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® 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® 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 stabilates 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 Schoenbechler, 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

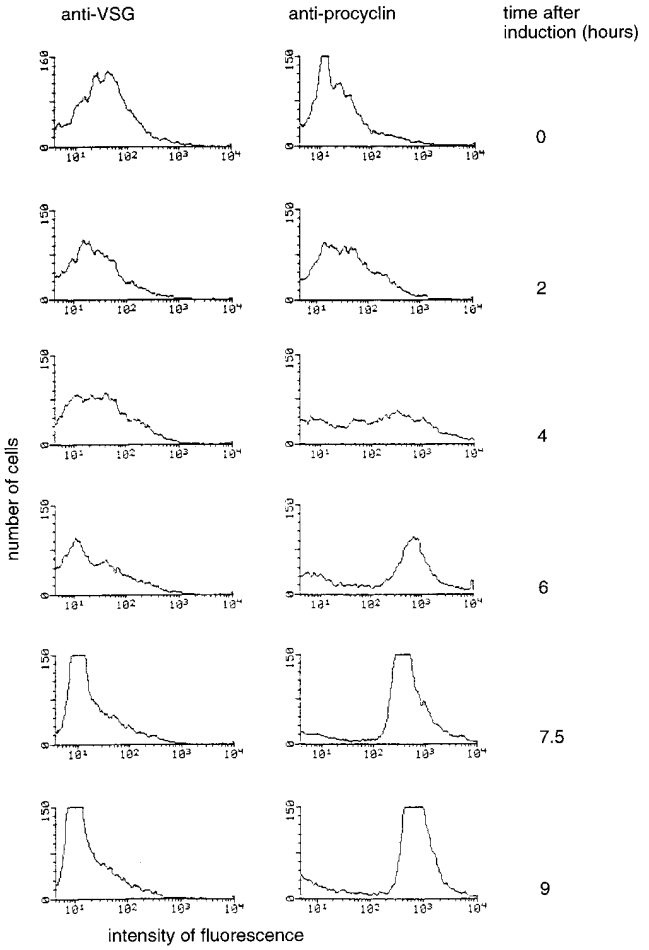

Figure 5. FACS® analysis showing the loss of VSG and the appearance of procyclin during the in vitro differentiation of *PLC*⁻ trypanosomes to procyclic forms.

with single trypanosomes, and stabilates were made 10–14 d later. Three mice were then infected using a single stabilate. The first mouse was used to prepare trypanosomes for immunofluorescence during the first peak of parasitemia. 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 immunofluorescence during the second peak of parasitemia. The results are shown in Fig. 7 *a*; the serum clearly recognizes 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 lysates. The triton lysates were then treated with recombinant GPI-PLC before Western blotting with anti-CRD (Fig. 7 *b*). Both populations are expressing readily detectable 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 trypanosomes was monitored in parallel with the *PLC*⁻ infections described above. Passage through a tsetse fly can

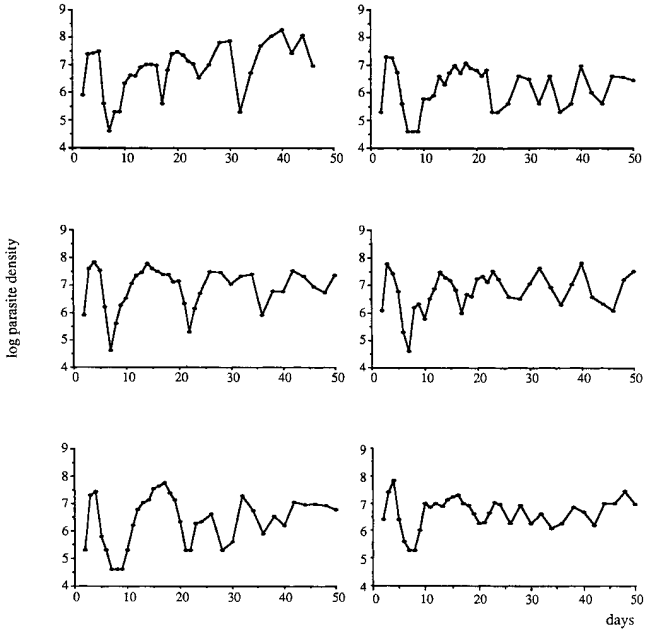

Figure 6. The course of infection with *PLC*⁻ trypanosomes in six individual mice. Parasite densities are expressed as $\log_{10}$ number of trypanosomes/ml of blood.

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-hygromycin^R gene construct integrated into the tubulin gene cluster (Berberof et al., 1995). Since isolation from tsetse flies, both of the trypanosome stocks had been amplified twice in immunosuppressed NMRI mice and stabilates prepared from the second mouse. These stabilates were used to infect immunosuppressed CFLP mice, and blood from these mice was used to establish persistent infections in matched sets of six CFLP mice. The innoculum for control and *PLC*⁻ trypanosomes was matched as closely as possible; both were collected while the parasitemia was rising and contained a preponderence of long slender and intermediate forms as opposed to stumpy forms. The differences between the two parasitemic profiles are illustrated in Fig. 8, which shows $\log_{10}$ geometric mean parasite density through time. Although each infection within a group gave the same basic profile, slight variation in the timing of peaks and troughs in individual mice means that the second and subsequent troughs are evident only by large standard error bars.

A parasitemia can be described in terms of the parameters that provide the column headings in Table I (Turner et al., 1995). Such characterization provided a clear demonstration that different trypanosome stocks are intrinsically different in the parasitemias they produce in chronic infections (Turner et al., 1995), and the same approach has been adopted here to compare *PLC*⁻ with control trypanosomes. The results are shown in Table I *a*, which also summarizes the results of two additional, parallel, infec-

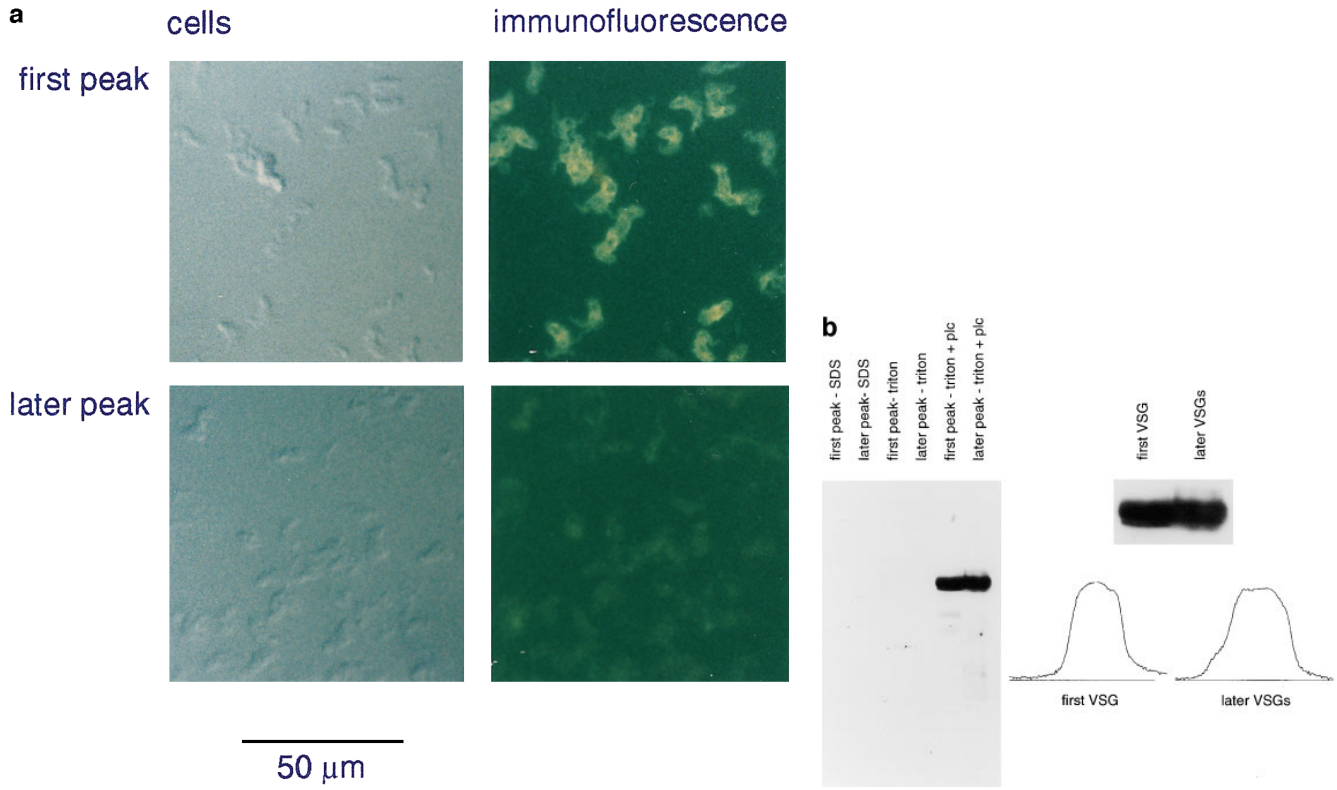

Figure 7. Antigenic variation in *PLC*⁻ trypanosomes. (*a*) Immunofluorescence of trypanosomes from the first and a successive peak of parasitemia using antiserum from a mouse that had just cleared the first peak. (*b*) A Western blot, probed with anti-CRD, showing the effect of GPI-PLC treatment of 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 μm.

tions with *PLC*⁻ trypanosomes. These were designed to investigate whether prolonged growth in mice and innoculum 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 1×10^6 trypanosomes as above and a second set with 1×10^7 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 1×10^8 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

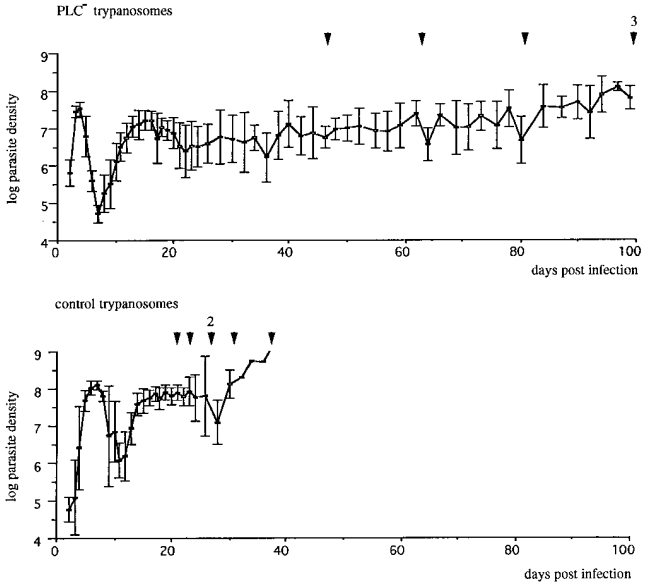

Figure 8. The course of infection in mice infected with control and *PLC*⁻ trypanosomes for comparison of parasitemias. Results are shown as geometric mean ± 2 standard errors, $n = 6$. Arrowheads indicate the survival time of individual mice.

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 $>1 \times 10^8$ (%)§	No. of days pd $<1 \times 10^7$ (%)§	Mean survival time
I*a*					
—					
Control	1	1.4×10^8	3.5 (17.5)	6.5 (32.5)	28.0
PLC⁻	3	4.1×10^7 ($P = 0.01$)	0	12.8 (64.2)	81.3 ($P = 0.01$)
PLC⁻22p	2	7.4×10^7 ($P = 0.01$)	1.2 (6.0)	11.2 (56)	72.8 ($P = 0.05$)
PLC⁻22p 1×10^7	1–2	7.2×10^7 ($P = 0.11$)	1.2 (6.0)	10.2 (50.8)	90.5 ($P = 0.01$)
I*b*					
—					
PLC⁻	3	2.4×10^7	0.2 (0.8)	14.7 (73.3)	74.3
PLC⁺	1–2	9.4×10^7 ($P = 0.01$)	2.2 (10.8)	9.7 (48.3)	56.7 ($P = 0.26$)

*A trough is defined as a drop in parasitaemia greater than one order of magnitude between adjacent time points (calculated for the first 40 d).
‡Geometric means of parasite density at the peak of the first wave of parasitaemia ($n = 6$, except for *PLC*⁻ 22p, where $n = 5$).
§The mean number of days on which parasite density (pd) was $\geq 1 \times 10^8$ cells per ml of blood, or $\leq 1 \times 10^7$ cells per ml of blood. These were determined for the first 20 d after infection and are expressed as percentages in parentheses.

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 innoculum 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 mfVSG 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 2×10^6 *PLC*⁺ trypanosomes was roughly equivalent to that in only 1.25×10^5 AnTat1.1 cells. Thus the *PLC*⁺ trypanosomes contain approximately one sixteenth of the wild-type, bloodsteam-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-CRD positive (Fig. 10). In the second assay, lysates of *PLC*⁺ and wild-type trypanosomes were assayed in vitro for release of tritium from [^{3}H]-myristyl VSG. The *PLC*⁺ trypanosome lysates contained an activity that released 15.5 pmol/min/mg protein or 31% of wild-type activity. There is a discrepency between the estimates of the amount of GPI-PLC protein (5–10% of wild-type) and the activity (30% of wild-type). At this stage it is not clear whether this is an artefact of the enzyme assay or due to some endogenous regulatory phenomenon.

Matched sets of six mice were infected with either *PLC*⁻ or *PLC*⁺ trypanosomes and the parasitemias monitored 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

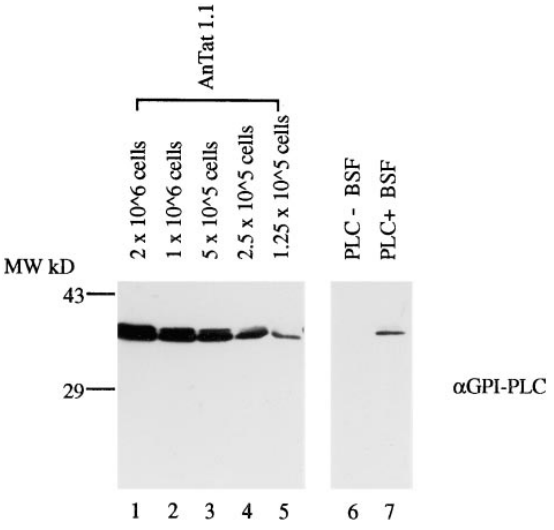

Figure 9. Expression of GPI-PLC protein in *PLC*⁺ trypanosomes. Lysates of bloodstream trypanosomes from three lines; AnTat 1.1, *PLC*⁻, and *PLC*⁺ trypanosomes were Western blotted and probed with anti–GPI-PLC. All samples are from the same blot and were probed simultaneously.

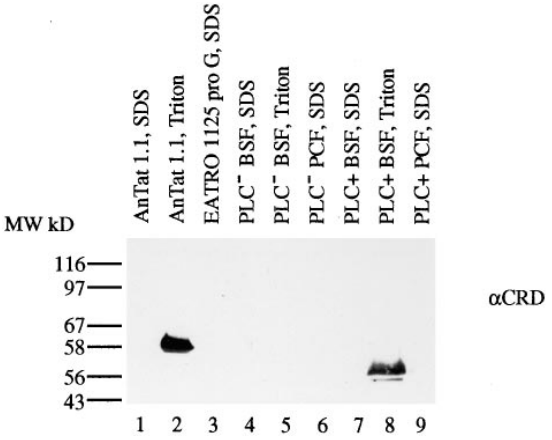

Figure 10. Presence of GPI-PLC activity in PLC^+ trypanosomes. Western analysis of bloodstream trypanosomes from four cell lines; AnTat 1.1, proG Anvers, PLC^- and PLC^+. Lysates were prepared by SDS or Triton lysis, Western blotted, and probed with anti-CRD. All samples are from the same blot and were 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 developmentally regulated expression of the *PLC* gene, as the deletion could be performed in a life cycle stage that does not express GPI-PLC. The success of the deletion strategy is in accord with previous findings that integration into the genome is the preferred mode of stable transformation in *T. brucei* and that integration is exclusively by homologous recombination. The high efficiency with which the desired recombinational event took place also supports the observation that constructs in which a drug-resistance marker is flanked by noncontiguous homologous sequences tend to lead to homologous replacement of the intervening sequences in the genome (Cruz and Beverley, 1990; Eid and Sollner-Webb, 1991; ten Asbroek et al., 1993).

The major find presented in this paper is that PLC^- trypanosomes are capable of completing an entire life cycle. PLC^- mutants were generated as procyclic trypanosomes and then subjected to cyclical transmission through tsetse flies. The ability of cells isolated from the salivary glands of tsetse flies to establish a chronic infection, at least in mice, suggests that the enzyme is inessential for the mechanism of switching from metacyclic to bloodstream VSG expression, or for the mechanism of antigenic variation. Finally, PLC^- bloodstream trypanosomes have been demonstrated to differentiate into procyclic trypanosomes both in vitro and in vivo with apparently normal kinetics.

An absence of any redundant activity that could substitute for the GPI-PLC had previously been suggested by quantitative immunoprecipitation of the mfVSG to sVSG activity in trypanosome lysates using a GPI-PLC–specific monoclonal antibody (Bülow and Overath, 1986). The inference that GPI-PLC is the only hydrolase catalyzing this conversion has been confirmed by the absence of any mfVSG-cleaving activity in PLC^- 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 lumenal leaflet of the ER (Vidugiriene 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 1×10^8 trypanosomes/ml contains roughly 100 μg/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 innoculum 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.

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