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Short communication

Virulence of *Trypanosoma brucei* strain 427 is not affected by the absence of glycosylphosphatidylinositol phospholipase C

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The infectious stages of *Trypanosoma brucei* are covered by a homogenous coat consisting of 10 million molecules of a glycosylphosphatidylinositol (GPI)-anchored variant surface glycoprotein (VSG) (for a review, see [1]). The bloodstream forms express a phospholipase, the GPI-specific phospholipase C (GPI-PLC), which has a predilection for cleaving the GPI anchor of cell-surface VSG when trypanosomes are disrupted (for a review, see [2]). During osmotic lysis, GPIPLC releases all of the cell-surface VSG within 5 min at 30°C, providing a convenient method for purifying soluble VSG (sVSG) [3]. GPIPLC also appears to be responsible for much of the degradation of the GPI precursor glycolipid A′ that occurs during lipid remodeling in vitro [4]. The normal cellular function of the GPIPLC is unknown, however, despite intensive study. The enzyme does not appear to be essential for normal differentiation to procyclic forms [5], when the surface VSG is released by proteolytic cleavage [6,7] and replaced by a small family of GPIPLC-resistant [8] GPI-anchored surface proteins that are characterized by glutamate–proline-rich repeats [9–12]. On the other hand, it has been reported that GPIPLC is necessary for the accelerated differentiation of pleomorphic trypanosomes that is induced by mild acid stress [18]. In this brief report, we document the generation of a GPIPLC null-mutant in the virulent 427 strain of *T. brucei*. These cells, which retain the T7 RNA polymerase (*T7RNAP*) and Tet repressor (*TETR*) cassettes (the genotype designation is Δ*gpiplc::T7RNAP–NEO*/Δ*gpiplc::*$P_{10\%T7}$*TETR–HYG*), will be useful for further biochemical and genetic studies of GPI synthesis and function, and for purification of GPI-linked proteins. The results also illustrate several issues concerning the use of genetically modified lines of *T. brucei*.

There have been three reports of *GPIPLC* disruption. In the first, it was found that a *GPIPLC* null mutant was not impaired in its ability to complete the natural life cycle, but the moderate virulence of this *T. brucei* line was further reduced [5]. A *GPIPLC* null mutation in a more virulent monomorphic cell line of *T. b. rhodesiense* also reduced its virulence in mice [13]. In our previous work, for reasons that could only be speculated upon, we were unable to obtain a null mutant in the highly virulent 427 line of *T. brucei*, although a conditional null mutant, expressing exceed-

Abbreviations: BLE, phleomycin resistance gene; DAG, diacylglycerol; GPI, glycosylphosphatidylinositol; GPIPLC, GPI phospholipase C; *GPIPLC*, GPIPLC gene; *HYG*, hygromycin B phosphotransferase gene; *NEO*, neomycin phosphotransferase gene; *T7RNAP*, T7RNA-polymerase gene; *TETR*, Tn*10*-encoded tetracycline-responsive repressor gene; VSG, variant surface glycoprotein.

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ingly low levels of GPIPLC, retained undiminished virulence in mice [14]. Virulent strains have a population doubling time of about 6 h: an inoculum of 5×10^5 trypanosomes overwhelms a mouse within 72 h. Infections with less virulent strains can persist for several weeks.

Stabilates (reference number Rockefeller University Molecular Parasitology (RUMP) 517) of blood from mice infected with the conditional null mutant [14] were provided to several laboratories wishing to study GPI pathways without the added complication of the presence of large amounts of a GPI-degrading enzyme. In the Englund laboratory, a sample of RUMP 517 was thawed and mixed with 2 ml of fresh HMI-9 medium [15] containing penicillin and streptomycin. The trypanosomes were not separated from red cells and the culture was left for 2 d at 37°C, when the cells grew to a density of about 2×10^6 ml^{-1}. Cells were diluted 1:1 and then, for 10 days, they were diluted 1:1 on alternate days, keeping them below 2×10^6 ml^{-1}. As they began to grow faster, during the next 2 weeks, the dilution rate was gradually increased to 1:20. After 6 weeks, rats were infected and stabilates prepared. After another 6 weeks of continuous culturing in vitro, GPIPLC activity was undetectable in lysates prepared from cells that had been incubated for 26 h with 0.1, 1, or 10 μg ml^{-1} tetracycline, using [^{3}H]myristate-labeled VSG as a substrate. When tested after another 2.5 weeks, all of these cells were killed within 1 day by 2.5 μg ml^{-1} phleomycin. These results suggested that the pLEW100-based [16] cassette containing the *BLE* gene coupled to the ectopic tetracycline-inducible *GPIPLC* [14] had been spontaneously eliminated from the conditional null mutant cells during prolonged cultivation in the absence of phleomycin, G418 and hygromycin. One of the stabilates that were prepared from the cells that were grown in rats after 6 weeks of cultivation in the absence of selection was transferred to culture in the presence of phleomycin. These cells were resistant to phleomycin and GPIPLC was inducible by tetracycline, as expected, suggesting that at least some of the cells that were cultured for 6 weeks in the absence of phleomycin maintained the ectopic copy, but none remained after 12 weeks.

We isolated three clones from the 12 week cultured cells and characterized them. All had the same genotype and phenotype, with respect to *GPIPLC* and drug resistance. A Southern blot of purified DNA digested with six different restriction enzymes showed no band that hybridized to a *GPIPLC* coding region probe (data not shown). Cells remained resistant to G418 and hygromycin, owing to the *NEO* and *HYG* genes that are coupled to *T7RNAP* and *TETR*, respectively [14], but died in the presence of phleomycin (2.5 μg ml^{-1}). Northern analysis (data not shown) and western blotting showed that the three clones continued to express VSG 221, as did the parental line. Lack of GPIPLC activity was demonstrated by the failure of these clones to release VSG under standard assay conditions (data for one of the clones are shown in Fig. 1, panel A).

Growth of the null mutant in mice was not noticeably different from the wild type: lethal parasitaemias of $>10^9$ ml^{-1} were reached in 72 h with a standard inoculum (5×10^5 trypanosomes). Infected mouse blood was frozen (stabilate reference number RUMP 528). There was no significant difference in the course of longer-term infections, in mice inoculated with ~ 5 trypanosomes. Wild-type, *GPIPLC* single-allele knockout, and *GPIPLC* null mutants achieved fatal parasitemias in 12–14 days, which is typical for *T. brucei* strain 427. We were interested to determine whether cultured null-mutant cells would differentiate to procyclic forms in vitro. They did not. The parental cells differentiated almost synchronously and will multiply indefinitely as procyclic forms. We then restored *GPIPLC* by stably transfecting the null mutant with the same regulated pLEW100-based ectopic cassette, pCO19, that was originally used to create the conditional null mutant [14]. Stably transformed phleomycin-resistant clones were obtained, which expressed GPIPLC after tetracycline induction (data for one of the clones is shown in Fig. 1, panels B and C). In several experiments, whether maintained in the presence or absence of tetracycline, these clones were also unable

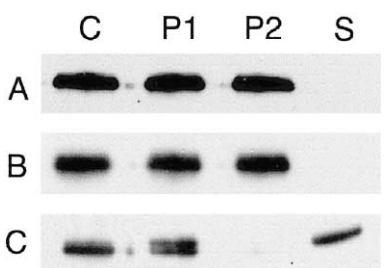

Fig. 1. GPIPLC activity of cell lines assayed by release of VSG 221. 1×10^7 cells were resuspended in 200 μl ice-cold water containing 0.1 mM TLCK and held on ice for 5 min [3]. After centrifuging at $3000 \times g$ for 5 min, the supernatant was discarded. The cell ghosts (P1) were resuspended in 200 μl 10 mM sodium phosphate buffer, pH 8.0, containing 0.1 mM TLCK. After incubation at 37°C for 15 min, the sample was centrifuged at $16\,000 \times g$ for 15 min to separate pellet (P2) and supernatant (S) fractions. Aliquots representing 2×10^4 cells were loaded per lane on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Amersham-Pharmacia). Tris-buffered saline plus 0.1% Tween-20 (TBST) containing 5% nonfat dry milk was used as blocking solution, incubated for 1 h at room temperature. The membranes were then incubated with rabbit *anti*-VSG 221 antibody (dilution 1:10 000), washed in TBST and incubated with horseradish peroxidase-conjugated goat-*anti*-rabbit antibody (dilution 1:10 000). Proteins were visualized with the ECL chemiluminescence substrate (Pierce) (10 s exposure). Lanes C, P1, P2 and S contain, respectively, whole cells, pellet after lysis at 0°C, and pellet and supernatant after GPIPLC activation at 37°C. Panel A, GPIPLC null mutant; B, null mutant after introduction of a regulated ectopic *GPIPLC*; C, as B but induced with tetracycline for 48 h.

to differentiate to procyclic forms under standard conditions, using DTM medium and cis-aconitate [17]. They died within 48 h. We attribute this result to the unexplained variation in differentiation ability that we see among different clones of the 427 strain of *T. brucei*, rather than to the genetic manipulations that were performed.

These results are significant for two reasons. Firstly, they provide virulent *GPIPLC* null-mutant trypanosomes that will be useful for further biochemical and genetic studies of GPI synthesis and function. Secondly, they emphasize that, in the absence of intrinsic selection for an essential gene, a pLEW100 cassette cloned into a rDNA locus can be spontaneously lost, presumably by homologous recombination among rDNA genes, of which there appear to be 15–20 copies in *T. brucei* 427 [19], distributed among chromosomes I, II, III and VIII [20]. Loss of the cassette from individual cells might be expected, in the absence of selection. What was more surprising is that the deletion presumably provided a slight selective advantage that allowed the null-mutant cells to slowly outgrow their parents. We can only speculate on possible reasons for this, but the most obvious possibility is that the introduction of the T7 promoter into some rDNA loci could be deleterious, perhaps due to transcription extending into regions downstream of the insertion site [21]. Unlike pLEW82 [21], pLEW100 [16] does not contain a downstream T7RNAP transcription terminator. The possibility that pLEW100 is only tolerated in a minority of rDNA loci could also explain why it is generally more difficult to obtain transfectants with this cassette, in contrast to pLEW82 (unpublished observations of several investigators). Finally, we wish to note that another laboratory was recently able to directly create a *GPIPLC* null mutant of *T. brucei* 427, using constructs previously described [5], without loss of virulence or the intermediate use of an ectopic copy (Mark Carrington, personal communication)

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