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Conditional expression of glycosylphosphatidylinositol phospholipase C in *Trypanosoma brucei*

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Abstract

Trypanosoma brucei glycosylphosphatidylinositol phospholipase C (GPIPLC) is expressed in the bloodstream stage of the life cycle, but not in the procyclic form. It is capable of hydrolyzing GPI-anchored proteins and phosphatidylinositol (PI) in vitro. Several roles have been proposed for GPIPLC in vivo, in the release of variant surface glycoprotein during differentiation or in the regulation of GPI and PI levels, but none has been substantiated. To explore GPIPLC function in vivo, tetracycline-inducible GPIPLC gene (*GPIPLC*) conditional knock-out bloodstream form and tetracycline-inducible *GPIPLC*-expressing procyclic cell lines were constructed. We were unable to generate *GPIPLC* null mutants. Cleavage of GPI-anchored proteins was abolished in extracts from uninduced conditional knock-outs and was restored upon induction. Despite the barely detectable level of GPIPLC activity in uninduced conditional knock-out bloodstream forms, their growth was not affected. GPI-protein cleavage activity could be induced in procyclic cell extracts, up to wild-type bloodstream levels. *Myo*-[^{3}H]inositol incorporation into [^{3}H]inositol monophosphate was about 14-fold lower in *GPIPLC* conditional knock-out bloodstream forms than in the wild type. Procyclic cells expressing *GPIPLC* showed a 28-fold increase in *myo*-[^{3}H]inositol incorporation into [^{3}H]inositol monophosphate and a 1.5-fold increase in [^{3}H]inositol trisphosphate levels, suggesting that GPIPLC may regulate levels of inositol phosphates, by cleavage of PI and phosphatidylinositol 4,5-bisphosphate.

Keywords: Glycosylphosphatidylinositol phospholipase C; Inositol phosphates; *Trypanosoma brucei*; Gene knock-out; Conditional expression

Abbreviations: AChE, Acetylcholinesterase; BLE, Phleomycin resistance gene; DAG, Diacylglycerol; GPI, Glycosylphosphatidylinositol; GPIPLC, GPI phospholipase C; *GPIPLC*, GPIPLC gene; *HYG*, Hygromycin B phosphotransferase gene; IP, Inositol 1-monophosphate; IP_2, Inositol 1,4-bisphosphate; IP_3, Inositol 1,4,5-trisphosphate; IP_4, Inositol 1,3,4,5-tetrakisphosphate; NEO, Neomycin phosphotransferase gene; PCR, Polymerase chain reaction; PFGE, Pulsed field gel electrophoresis; PLC, Phospholipase C; PI, Phosphatidylinositol; PIP_2, Phosphatidylinositol 4,5-bisphosphate; *T7RNAP*, T7 RNA-polymerase gene; Tc, Tetracycline; *Tet*R, Tn*10*-encoded Tetracycline-responsive repressor; UTR, Untranslated region; VSG, Variant surface glycoprotein.

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1. Introduction

Trypanosoma brucei bloodstream forms are covered by a homogeneous coat of glycosylphosphatidylinositol (GPI)-anchored variant surface glycoprotein (VSG) (for a review, see [1]) and contain a GPI phospholipase C (GPIPLC), which cleaves the VSG GPI-anchor, forming diacylglycerol (DAG) and 1,2-cyclic phosphate on the inositol ring [2–4]. The enzyme is present only in the bloodstream and metacyclic stages of the parasite's life cycle, in which *T. brucei* is covered with a VSG coat [5]. Bloodstream forms differentiate into procyclic forms after ingestion by the insect vector, or in suitable culture systems. Procyclic forms contain no detectable enzyme activity [5], and very low levels of GPIPLC mRNA [6].

GPIPLC is undoubtedly responsible for the rapid cleavage of membrane-bound VSG (mfVSG) when cells are lysed by hypotonic shock or detergents [6]. However, the function of the enzyme under physiological conditions remains undiscovered. In particular, GPIPLC does not play an obligatory role in the release of surface VSG during differentiation to procyclic forms [7,8], which appears to be attributable to proteolytic cleavage [9–11]. Further data suggest that VSG is released through synthesis or activation of a developmentally regulated proteinase, shortly after treating bloodstream forms with the Krebs cycle intermediates citrate and/or *cis*-aconitate, and shifting their culture temperature to 27°C, to induce differentiation [10]. Under these conditions, differentiation does not require GPIPLC. However, low-pH-induced differentiation, whose physiological relevance is unclear, would not occur in *GPIPLC* null mutants [8]. Thus, any biological role for GPIPLC as a VSG lipase remains elusive and the stage-specific co-expression of VSG and GPIPLC may only be a coincidence.

The sub-cellular localization of GPIPLC remains uncertain. Based upon sub-cellular fractionation and immunofluorescence experiments, the enzyme appears to reside on the cytoplasmic side of otherwise uncharacterized intracellular vesicles [12]. This location presents a major topological impediment to any role of GPIPLC in VSG shedding in vivo, but would be consistent with a role for GPIPLC in phosphatidylinositol (PI) or GPI metabolism.

Purified *T. brucei* GPIPLC, or recombinant *T. brucei* GPIPLC expressed from *Escherichia coli*, can cleave PI to inositol 1-monophosphate (IP) in vitro and during trypanosome lysis [13], like a prokaryotic PI-specific phospholipase C (PLC) [14,15]. PI is a key intermediate in at least two important pathways. It is the precursor of the inositol phosphates cascade leading to phosphatidylinositol bisphosphate (PIP_2), which gives rise to the secondary intracellular messengers, inositol 1,4,5-trisphosphate (IP_3) and DAG. These two metabolites are responsible for activation of signal transduction pathways related to Ca^{2+} [16] and to Ca^{2+}-dependent and Ca^{2+}-independent protein kinase C [17]. PI is also a constituent of protein GPI anchors [18] and a precursor of other complex lipids. Biochemical data on the metabolism of inositol phosphates in *Crithidia fasciculata* showed that *myo*-[^{3}H]inositol (Ins) is incorporated into PI by phosphatidylinositol synthase, in a two-step reaction [19]. Presumably, such activity may also be present in *T. brucei*, since *myo*-[^{3}H]Ins incorporation into different phosphoinositides has been detected in bloodstream and procyclic cells [20]. PI turnover is considered to be involved in the regulation of mammalian cell growth. The enzymes for PI turnover include phospholipase C (PLCγ), PI 4-kinase and phosphatidylinositol synthase. It has been suggested that PIP_2 hydrolysis by PLCγ is involved in the regulation of serum-induced S-phase induction in mammalian cells [21] and that PI synthesis is involved in growth control of yeast [22].

In the current study, we manipulated the expression of *T. brucei* GPIPLC to probe its functions in vivo. For this purpose, we generated tetracycline-inducible (Tc-inducible) *GPIPLC* conditional knock-out bloodstream form and *GPIPLC*-expressing procyclic *T. brucei*.

2. Materials and methods

2.1. Materials

All reagents were from Sigma, unless otherwise stated. dCTP-α[^{32}P] (3000 Ci mmol^{-1}) was purchased from Dupont-NEN and *myo*-[^{3}H]inositol (80 Ci mmol^{-1}) from Amersham Pharmacia Biotech. L-α-[*myoinositol*-2-^{3}H]phosphatidylinositol (13 Ci mmol^{-1}) was purchased from DuPont-NEN. Bovine erythrocyte acetylcholinesterase (AChE) was purified by affinity chromatography, as described previously [23]. G418 (Geneticin®) was from GIBCO-BRL, hygromycin-B was from Boehringer Mannheim, and phleomycin was from CayLa (France). The liquid scintillation cocktail Ready Safe™ was from Beckman.

2.2. Trypanosomes and culture conditions

T. brucei monomorphic strain 427 [24] procyclic and bloodstream-form (Molteno Institute Trypanozoon antigenic type 1.2 (MITat 1.2) clone 221a) cells were used in these studies. Bloodstream forms were cultured in HMI-9 medium at 37°C [25] and procyclic forms in SDM-79 medium at 27°C [26]. Transgenic procyclic cell line 29-13 clone 6 and transgenic bloodstream cell line 90-13 clone 4 constitutively express T7 RNA polymerase (T7RNAP) and Tet repressor (TetR) [27]. Genotypes [28] of *T. brucei* bloodstream lines used were: parental strain 90-13 (*T7RNAP::NEO* P_{T7}*::TETR::HYG*); *GPIPLC* one-allele knock-out 17 (*Δgpiplc::T7RNAP::NEO*); *GPIPLC* conditional knock-out 17-19-18 (P^{Ti}_{parp}*::GPIPLC::BLE Δgpiplc::T7RNAP::NEO*/ *Δgpiplc::*$P_{T7::}$*TETR::HYG*). Genotypes of *T. brucei* procyclic lines used were: parental strain 29-13 (*T7RNAP::NEO* $P_{T7::}$*TETR::HYG*); Tc-inducible *GPIPLC* 29-13-19 (P^{Ti}_{parp}*::GPIPLC::BLE T7RNAP::NEO* $P_{T7::}$*TETR::HYG*).

2.3. Transfection of trypanosomes

Plasmid DNA for transfection was prepared using anion exchange columns (Qiagen). Bloodstream and procyclic forms were transformed with 10 μg of plasmids linearized by *Not* I digestion, as previously described [29]. Each electroporation of transfected bloodstream cells was immediately aliquoted in 24-well plates with HMI-9. Antibiotics were added to the bloodstream culture medium 16 h after electroporation, to final concentrations of 2.5 μg ml^{-1} G418, 5 μg ml^{-1} hygromycin B, and 2.5 μg ml^{-1} phleomycin. All bloodstream cell populations used in this study were cloned by limiting dilution in vitro. Antibiotics were added to the SDM-79 medium 16 h after the procyclic cell electroporations, to final concentrations of 15 μg ml^{-1} G418, 50 μg ml^{-1} hygromycin B, and 2.5 μg ml^{-1} phleomycin. Procyclic-form trypanosomes were serially diluted in microtiter plates with conditioned medium. For all subsequent assays, drug-resistant clones were grown in the absence of antibiotics, unless otherwise stated.

2.4. Construction of targeting vectors

Polymerase chain reaction (PCR) amplification of *GPIPLC* coding sequence (GenBank M27301; nucleotides 80–1167), *GPIPLC* 5′ untranslated region (UTR) (GenBank M27301; nucleotides 1–93), and *GPIPLC* 3′ UTR (GenBank M27301; nucleotides 1153–1328) from genomic DNA of *T. brucei* strain 427 was performed by using the oligonucleotide primers OC56 (5′-GGAAGCTTTCTTTGTAATGTTTGGTGGTGT-3′) and OC57r (5′-CCGGATCCTTTTTATGACCTTGCGGTTTGG-3′), OC52 (5′-GGGCGGCCGCGTGCGTGTGGGTGTCTGAGA-3′) and OC53r (5′-AAGTATACACGCGTCTCGAGTCATCTATTTAAACACCACCAAACATTACAAAG-3′), and, OC54 (5′-GGGAAAGATCTAGAGCAAGGTCATAAAAATGTGGG-3′) and OC55r (5′-CCCAGGCCTGCGGCCGCTTCCAACGTCATTGTCGCCC-3′), respectively. All PCR-amplified products were confirmed by sequencing. Plasmid pCO19 was constructed to express *GPIPLC* in a Tc-dependent way. The 1076-bp PCR product from OC56–OC57r was digested with *Hin*d III and *Bam*H I, and ligated into the similarly digested vector pLew79 [27], replacing *LUCIFERASE*, yielding the plasmid pCO19, constructed to target the rRNA spacer (Fig. 1B). Plasmid pCO17 was constructed to knock-out one

allele of the *GPIPLC* gene (Fig. 1A). The *GPIPLC* 5′ UTR 125-bp PCR product from OC52 and OC53r was digested with *Not* I and *Mlu* I, and inserted in a similarly digested vector pLew13 [27], to yield pCO16. The *GPIPLC* 3′ UTR 190-bp PCR product from OC54 and OC55r was digested with *Xba* I and *Stu* I, and ligated to a similarly digested vector pCO16, to yield pCO17. Plasmid pCO18 was constructed to knock-out the second allele of the *GPIPLC* gene (Fig. 1A). A *Xho* I–*Stu* I 2490-bp fragment from plasmid pLew90 [27] was cloned into an *Xba* I, blunt-ended *Xho* I 4370-bp fragment from plasmid pCO17, to generate pCO18. This plasmid contains the Tn5 hygromycin phosphotransferase gene (*HYG*), flanked upstream by the Tetracycline repressor (*Tet*R) open reading frame (GenBank TRN10TETR; nucleotides 903–1526), and preceded by a 10% strength T7RNAP promoter [27]. Cloning junctions and all PCR-amplified fragments were sequenced at The Rockefeller University Shared Technology Resource Center.

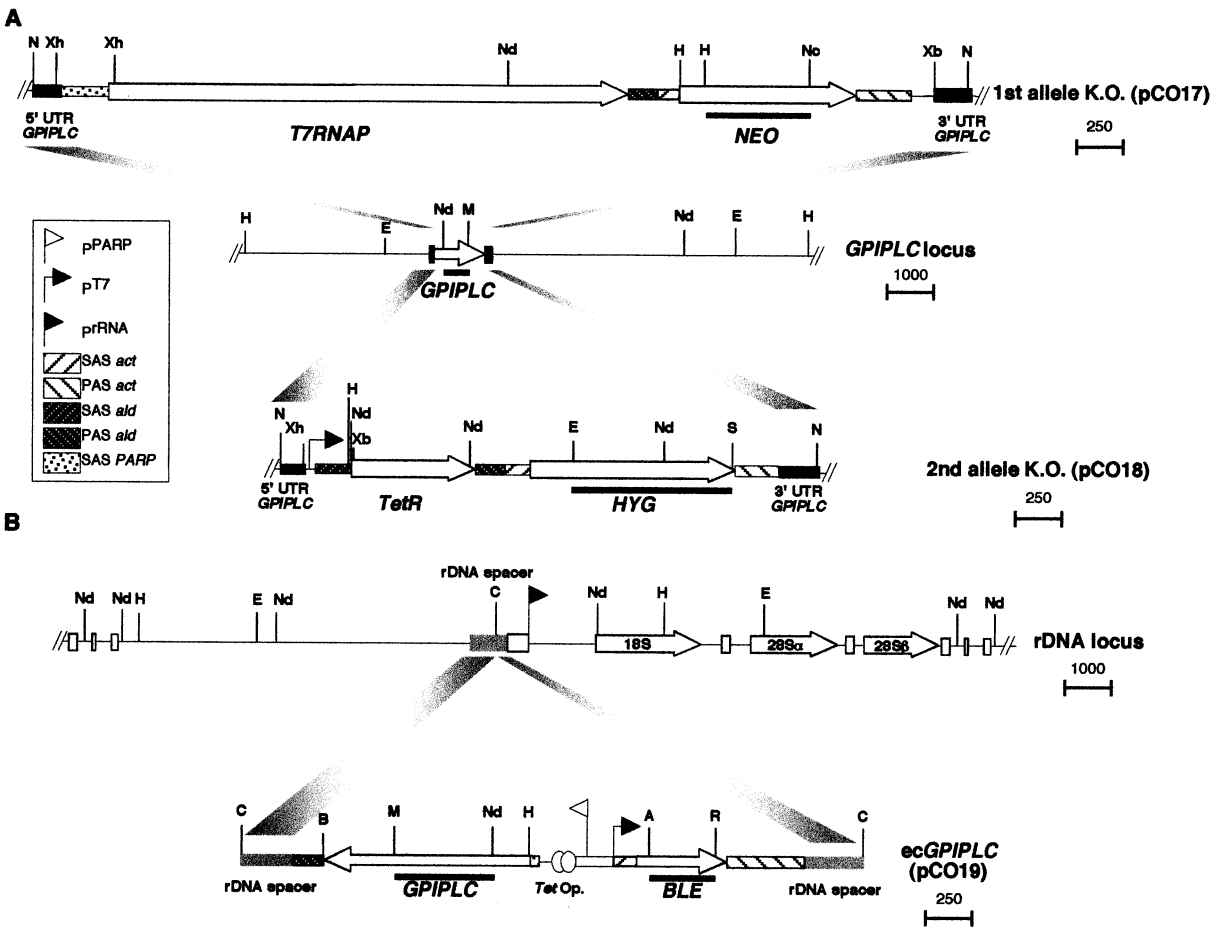

Fig. 1. Strategy to generate inducible *GPIPLC* cell lines. Constructs and targeting strategy used for (A) the replacement of *GPIPLC* endogenous alleles and (B) for the stable expression of an ectopic copy of *GPIPLC* (*ecGPIPLC*) in a rDNA locus of *T. brucei*. The black arrow indicates a *T7RNAP* promoter, the open arrow indicates a Tc-inducible *PARP* promoter, and the grey arrow indicates a *rRNA* promoter. Solid bars under the constructs indicate position of probes used for Southern and Northern analyses. Abbreviations: act, actin; adl, aldolase; PARP, procyclic acidic repetitive protein; PAS, polyadenylation sequence; SAS, splice-acceptor sequence. Restriction enzyme abbreviations: A, *Aat* II, B; *Bam*H I; C, *Cla* I; E, *Eco*R I; H, *Hin*d III; M, *Mlu* I; N, *Not* I; Nc, *Nco* I; Nd, *Nde* I; R, *Rsr* II; S, *Sca* I; Xb, *Xba* I; Xh, *Xho* I.

2.5. RNA and DNA analysis and probes

Total RNA or DNA from trypanosomes was isolated using RNAstat60™ (Tel-Test 'B', Friendswood, TX) or DNAzol® (Molecular Research Center, Cincinnati, OH). Parasite blocks for pulsed field gel electrophoresis (PFGE) were prepared as previously described [30] at a concentration of 4×10^8 trypanosomes ml^{-1}. Northern and Southern analysis were carried out according to standard protocols [31]. RNA and DNA were cross-linked to a Hybond N^+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ) in a UV Stratalinker-1800 (Stratagene, La Jolla, CA). Probes used for Southern and Northern analyses were a 523-bp *Nde* I–*Mlu* I fragment from the *GPIPLC* gene, a 357-bp *Rsr* II–*Aat* II fragment from the *BLE* gene, a 727-bp *Eco*R I–*Sca* I fragment from the *HYG* gene, a 555-bp *Hin*d III–*Nco* I fragment from the *NEO* gene, a 700-bp *Hin*d III–*Eco*R I fragment from the *TUBULIN* gene, and a 770-bp *Eco*R I fragment from the procyclic acidic repetitive protein (*PARP*) coding sequence. Probes were labeled by using the Prime-It II® kit (Stratagene) and purified with NucTrap® Probe Purification Columns (Stratagene). All hybridizations and washes were performed at 65°C in a hybridization oven (Hybaid), using the QuikHyb® system (Stratagene). Washes were for 30 min in $1 \times$ SSC ($1 \times$ SSC: 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS. Final washes were at high stringency ($0.1 \times$ SSC, 0.1% SDS). DNA and RNA sizes were estimated according to the migration of 1 kb DNA Ladder (GIBCO-BRL), and 0.24–9.5 kb RNA ladder (GIBCO-BRL), respectively. Northern hybridization signals were quantified by phosphorimager analysis and the Imagequant™ Software (Molecular Dynamics). Chromosomal DNA separations were performed in 0.8% agarose gels, in a rotating agarose gel electrophoresis apparatus (Stratagene). The program consisted of three windows: a 20-s pulse at 150 V for 5 h, followed by a 500-s pulse at 80 V for 35 h, then a 1500-s pulse at 80 V for 25 h. The rotating angle was 105°, and a $0.5 \times$ Tris-borate–EDTA re-circulating buffer was run at 12°C. DNA size was estimated from the migration of *Hansenula wingeii* PFGE size standard (BIO-RAD).

2.6. GPI hydrolysis assays

AChE from bovine erythrocytes, a GPI-anchored protein, was used as substrate for measuring GPI hydrolysis, as previously described [13], in a buffer containing 50 mM Tris, pH 8.0, and 2 mM EDTA. The extent of GPI-anchor hydrolysis was determined by measuring the relative amount of AChE in the aqueous phase after partitioning the reaction mixture in Triton X-114 [32]. Assays using extracts from trypanosomes were performed at 37°C. AChE activity was determined as previously described [33]. Each experiment contained control samples incubated in the absence of extracts. The corresponding controls have been substracted from experimental values. Average values from duplicate determinations performed in several independent experiments showed a variability of $< 10\%$. A GPIPLC assay using [^{3}H]mfVSG as a substrate was performed as previously described [34].

2.7. PI hydrolysis analysis

PI hydrolysis was performed essentially as previously described [13] at 37°C for trypanosome extracts in the buffer described for AChE hydrolysis, unless otherwise noted. The extent of [^{3}H]PI hydrolysis was measured by determining the distribution of radioactivity between the aqueous and organic phases after low speed centrifugation. The sum of radioactivity in the aqueous and organic phases, representing hydrolyzed and non-hydrolyzed [^{3}H]PI, respectively, was constant in all samples and corresponded to the amount of [^{3}H]PI added to the incubation mixtures (recovery of radioactivity was $> 90\%$). Each experiment contained control samples incubated in the absence of extracts. All numbers were expressed after substraction of the corresponding controls. Average values from duplicate determinations, performed in several independent experiments, showed a variability of $< 10\%$.

2.8. Labeling and extraction of water-soluble inositol phosphates

Labeling of trypanosomes with *myo*-[^{3}H]Ins was performed as described previously [35], with modifications. Exponentially growing bloodstream or procyclic parasites were centrifuged and resuspended in 5 ml serum-free HMI-9 (7.2 mg l^{-1} Ins) or serum-free and hemin-free SDM-79 (5 mg l^{-1} Ins) [20], respectively, supplemented with 50 mM glucose and 50 mg ml^{-1} BSA, at 5×10^6 parasites ml^{-1}. Bloodstream and procyclic forms were incubated with 10 μCi ml^{-1} of *myo*-[^{3}H]Ins (80 Ci $mmol^{-1}$) for 18 h at 37 or 27°C, respectively, and optionally supplemented with 1 or 2.5 μg ml^{-1} Tc, respectively. Parasite counts performed at the end of incubation time confirmed that cells had multiplied at the normal rate. [^{3}H]IP_1 and [^{3}H]IP_3 produced by the cells was isolated chromatographically on Dowex AG® 1-X8 anion exchange resin (BIO-RAD) in the formate form (200–400 mesh), as recommended by *myo*-[^{3}H[inositol manufacturer (Amersham Pharmacia Biotech) and described elsewhere [36]. Free Ins was not retained on the column. Glycerophosphoinositol and 1:2-cyclic phosphate were eluted with 5 mM-sodium tetraborate plus 60 mM ammonium formate, as described [35]. Standards of IP, inositol 1,4-bisphosphate (IP_2), IP_3 and inositol 1,3,4,5-tetrakisphosphate (IP_4) (Amersham Pharmacia Biotech) eluted at 0 M ammonium formate, 0.3–0.4, 0.5–0.6, 0.7–0.8 and 0.9–1.0 M ammonium formate, respectively. A standard of the water-soluble GPI glycan (Man_3GlcN-Ins) eluted at 60 mM ammonium formate.

3. Results

3.1. Generation of GPIPLC conditional knock-out bloodstream forms

We designed a conditional knock-out strategy (Fig. 1A), to avoid potential lethality, when trying to obtain *GPIPLC* null mutants in the monomorphic strain 427, and to be able to conditionally restore GPIPLC activity under in vivo conditions without further genetic manipulation. One allele was replaced with a cassette carrying *T7RNAP* and *NEO* (construct pCO17) using the *GPIPLC* 3′ and 5′ UTR as targeting sequences. Recombinant parasites were selected with G418 and analyzed by Southern blot (Fig. 2B) and PFGE (Fig. 2A). A second construct (pCO19), carrying *GPIPLC* driven by a Tc-inducible *PARP* promoter, was targeted to the rRNA spacer of the *GPIPLC* one-allele knock-out (Fig. 1B). Several G418 and phleomycin-resistant clones from independent transformations had inserted the cassette in at least two different rDNA loci (Fig. 2A). The remaining endogenous *GPIPLC* allele was replaced in those clones with a cassette expressing *TetR* and *HYG*, both driven by a *T7RNAP* promoter (construct pCO18). Recombinant parasites resistant to all three inhibitors were selected in the presence of 100 ng ml^{-1} Tc. The correct replacement of *GPIPLC* by the markers and the insertion of the ectopic copy in the bloodstream-form cell line 17-19-18 were confirmed by PFGE (Fig. 2A) and Southern blot (Fig. 2B).

In many attempts and using alternative strategies, no clones were obtained with lower amounts of Tc, or in the absence of Tc, suggesting that the absence of GPIPLC is lethal, or severely limits growth, in *T. brucei* strain 427. However, uninduced *GPIPLC* conditional knock-out bloodstream cells grew as well as wild-type cells, in vitro or in vivo. The mean survival time of mice infected with 5–10 trypanosomes of this virulent strain was 14 days, whether or not GPIPLC was induced (data not shown).

3.2. Tc-induced transcription of GPIPLC in procyclic forms

To express *GPIPLC* in procyclic cells, the native *GPIPLC* open reading frame was expressed in the *T7RNAP-TetR*-expressing procyclic cell line 29-13, under the control of a Tc-inducible *PARP* promoter [27], as depicted in Fig. 1B. Detectable levels of *GPIPLC* mRNA became evident as early as 2 h after Tc induction, reaching a maximum by 24 h (Fig. 3A). Quantification, by phosphorimaging, showed that the 1.4-kb band, corresponding to the ectopic copy, was sevenfold higher than the 3.4-kb bloodstream-form wild-

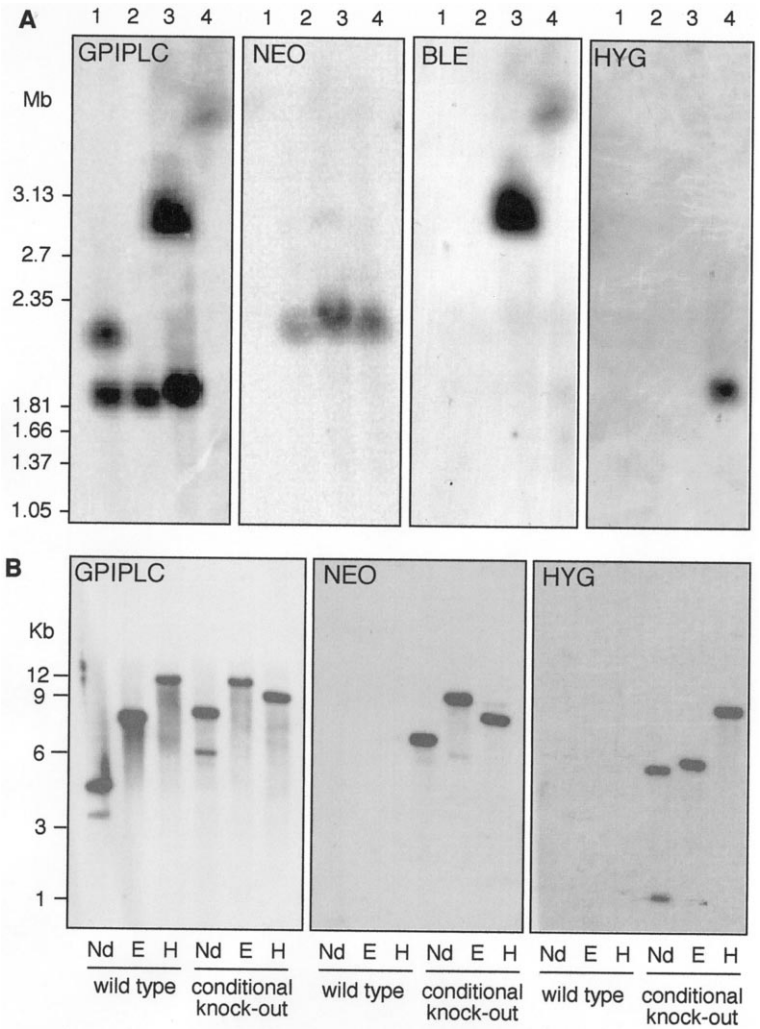

Fig. 2. Chromosomal and Southern analysis of *GPIPLC* conditional knock-out cell line. (A) Lane 1, wild-type cells; lane 2, *GPIPLC* one allele knock-out cell line (clone 17.1); lane 3, *GPIPLC* one allele knock-out cell line with an ectopic *GPIPLC* copy introduced into the rDNA spacer (clone 17.1-19.2); lane 4, final *GPIPLC* conditional double knock-out cell line (17.1-19.3-18.1). Unfortunately, the parental line used for the second allele knockout (lane 4) was lost, and no other clone contained the ectopic copy in the large chromosomal rDNA locus, which is why a different clone, where the ectopic copy inserted into a smaller chromosome, is shown in lane 3. (B) Lanes 1–3, wild-type bloodstream cells; lanes 3–6, *GPIPLC* conditional knock-out bloodstream cells. The same blot was hybridized with successive probes. Enzyme abbreviations as in Fig. 1.

type RNA. *GPIPLC* expression was tightly regulated. In the absence of Tc, *GPIPLC* mRNA was undetectable by Northern blot analysis and comparable with the signal obtained for wild-type procyclic cells (Fig. 3A).

3.3. Regulated expression of GPIPLC in a conditional knock-out bloodstream cell line

A *GPIPLC* conditional knock-out bloodstream clone was analyzed for its steady-state RNA levels

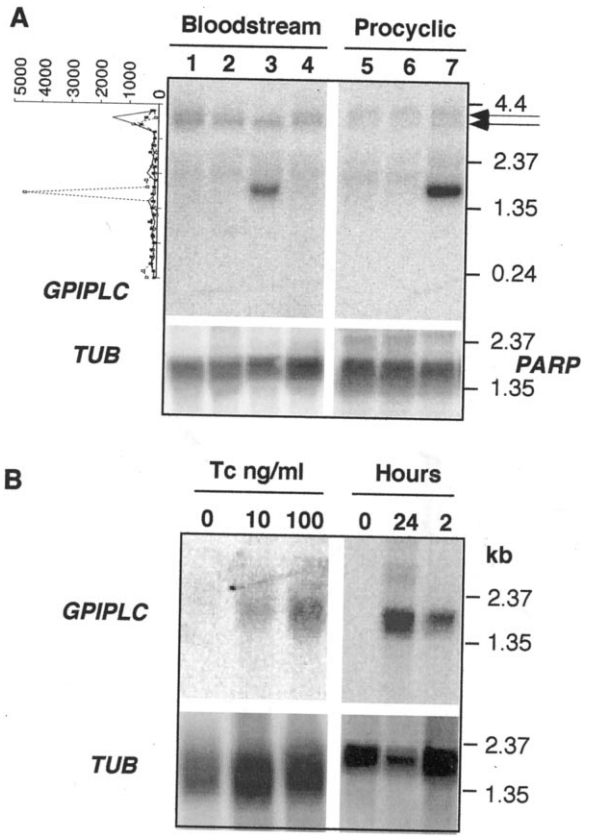

Fig. 3. Induction of *GPIPLC* mRNA in conditional knock-out bloodstream cells and GPIPLC expressing procyclic cells. (A) Induction in bloodstream and procyclic forms. Probes are indicated to the left of each panel. The *TUB* or *PARP* probes served as loading controls. Lane 1, wild-type bloodstream cells; lane 2, non-induced *GPIPLC* conditional knock-out bloodstream clone 17-19-18; lane 3, 24 h induction with 1 μg ml^{-1} Tc of *GPIPLC* conditional knock-out bloodstream clone 17-19-18; lane 4, *GPIPLC* one allele knock-out bloodstream clone 17; lane 5, parental procyclic 29-13 cell line; lane 6, non-induced *GPIPLC* expressing procyclic clone 29-13-19; lane 7, 24 h induction with 1 μg ml^{-1} Tc of *GPIPLC* expressing procyclic clone 29-13-19. Arrows indicate position of 3.4 kb doublet band referred to in the text. Insert represents quantification of gel profile from (—) wild-type bloodstream cells, (—■—) *GPIPLC* conditional knock-out non-induced cells, (···□···) *GPIPLC* conditional knock-out Tc-induced cells. (B) Induction kinetics in bloodstream forms. Results are shown for *GPIPLC* conditional knock-out bloodstream clone 17-19-18, when cultures were induced for 24 h with increasing amounts of Tc (left panel) or when cultures were induced with 1 μg ml^{-1} Tc for different times (right panel).

of *GPIPLC*. These cells showed a very tight expression of the 1.4-kb band corresponding to the ectopic *GPIPLC* copy: no signal was detectable in the absence of Tc (Fig. 3A). The 1.4-kb band became detectable at 10 ng ml^{-1} Tc, exhibiting a saturated signal at 1 μg ml^{-1} Tc (Fig. 3B). The levels of the 1.4-kb ectopic copy after 2 and 24 h of 1 μg ml^{-1} Tc stimulation were eight- and 14-fold above background, respectively (Fig. 3B). In bloodstream cultures stimulated with 10 or 100 ng ml^{-1} Tc for 24 h, expression of the 1.4-kb band was three- and sevenfold higher than the background, respectively. A 3.4-kb duplex band is normally detectable in wild-type bloodstream forms, although the open reading frame is only 1.4 kb (Fig. 3A). When both *GPIPLC* alleles were replaced, the higher band of the 3.4-kb duplex disappeared (Fig. 3A). The lower band of the 3.4-kb signal remained visible under Northern analysis when both alleles of *GPIPLC* were knocked-out and it was also visible in procyclic parental cells (Fig. 3A), suggesting the existence of a mRNA cross-hybridizing with the probe. This cross-hybridization may be explained by genes sharing some sequence similarity with the *GPIPLC* open reading frame, but no GPIPLC activity can be detected in procyclic cells [6] and was barely detectable in our uninduced conditional knock-outs (see later), so this cross-reacting RNA is very unlikely to encode a *GPIPLC* paralog.

3.4. Inducible GPI- and PI-hydrolyzing activity in GPIPLC bloodstream conditional knock-outs and GPIPLC-expressing procyclic cell extracts

GPI- and PI-hydrolyzing activities were measured in cell extracts using AChE, mfVSG and PI as substrates (Fig. 4A). At 1 μg ml^{-1} Tc, maximum GPIPLC activity was observed after 24 h, in both conditional knock-out bloodstream and inducible procyclic forms. The Tc induction system generally gives rise to some initial overshoot in expression levels, as seen here, before equilibrium between synthesis and turnover of mRNA and/or protein is attained. In the GPIPLC conditional knock-out bloodstream forms, GPI-hydrolyzing activity reached 45–50% of wild-type bloodstream

form levels, while PI-hydrolyzing activity reached 65–75% of wild-type extracts. In the GPIPLC-expressing procyclic cells, GPI- and PI-hydrolyzing activities reached 90–105 and 60–65%, respectively, of the activities in wild-type bloodstream form cells.

GPI-hydrolyzing activity was undetectable in the Tc-inducible bloodstream form or procyclic cultures at Tc doses below 1 ng ml^{-1}. Maximum AChE, mfVSG and PI hydrolysis was induced with 10–100 ng ml^{-1} Tc in bloodstream forms (Fig. 4B) and with 100 ng ml^{-1} Tc in procyclics (Fig. 4C), with the exception that maximum AChE hydrolysis required higher Tc levels in procyclics. Previous experiments showed that maximum expression of Luciferase occurred at 10 ng ml^{-1} Tc in both life life-cycle stages [27]. This suggests that the relationship between mRNA and protein levels, or enzyme activity towards different substrates, may be influenced by the nature of the specific transcript, the necessity for post-translational modification [37], and the parasite life-cycle stage.

Tc-induced GPIPLC was responsible for 75% of PI hydrolysis in extracts from both bloodstream (Fig. 4B) and procyclic forms (Fig. 4C). The source of the other 25% of PI hydrolysis, under our assay conditions, has not been investigated. The ratio of PI to GPI hydrolysis in vitro differed about twofold between bloodstream and procyclic forms (Fig. 4A), which might be due to differences in post-translational modifications between the two life-cycle stages, such as the acylation that has been detected in bloodstream-form GPIPLC [37].

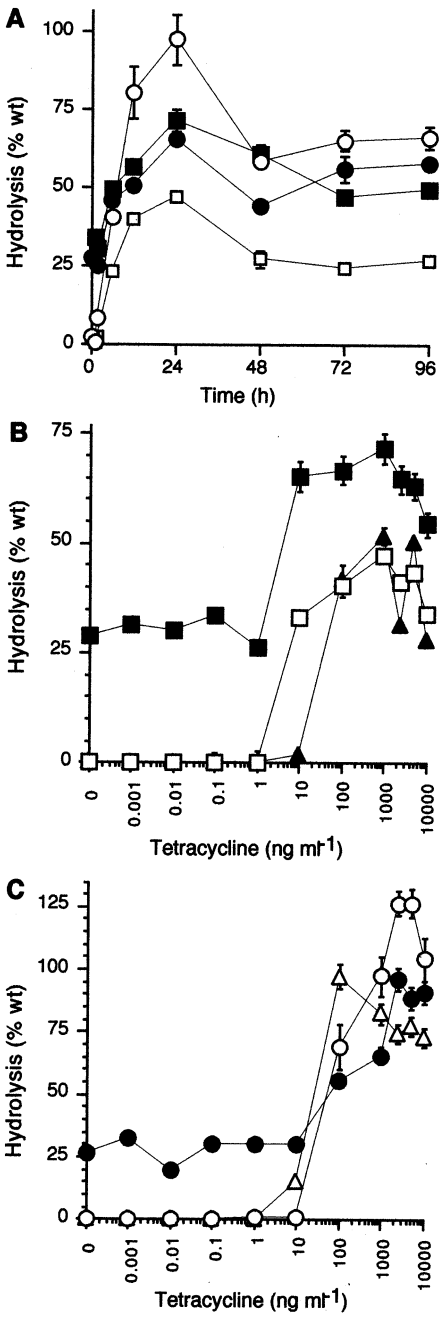

Fig. 4. (*Continued*)

Fig. 4. Inducible GPIPLC hydrolyzes GPI-anchored proteins and PI in vitro. (A) Time course of AChE and PI hydrolysis when cultures were supplemented with 1 μg ml^{-1} Tc ($n = 3$) (B) Tc dose-dependency curve for bloodstream cell extracts ($n = 3$). (C) Tc dose-dependency curve for procyclic cell extracts ($n = 2$). Cultures were stimulated for 24 h with increasing amounts of the drug. Activity values were related to a reference value of 100% of either GPI- or PI-hydrolyzing activities from *GPIPLC* wild-type bloodstream cell extracts. ○, AChE-hydrolyzing activity by *GPIPLC*-expressing procyclic cell extracts; ●, PI-hydrolyzing activity by *GPIPLC*-expressing procyclic cell extracts; □, AChE-hydrolyzing activity by *GPIPLC* conditional knock-out bloodstream cell extracts; ■, PI-hydrolyzing activity by *GPIPLC* conditional knock-out bloodstream cell extracts; ▲, mfVSG-hydrolyzing activity by *GPIPLC* conditional knock-out bloodstream cell extracts; △, mfVSG-hydrolyzing activity by *GPIPLC*-expressing procyclic cell extracts.

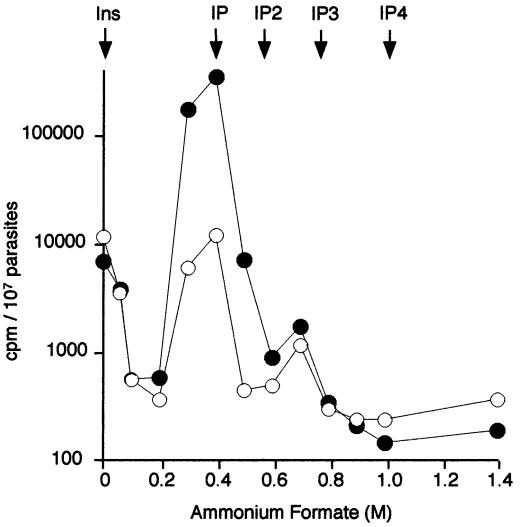

Fig. 5. Incorporation of *myo*-[^{3}H]inositol into inositol phosphates in *GPIPLC*-expressing procyclic cells. Elution profiles of water-soluble extracts from Dowex AG® 1-X8 anion-exchange columns. ○, non-induced; ●, induced with 2.5 μg ml^{-1} Tc for 24 h, including *myo*-[^{3}H]Ins incorporation time. Radioactivity measured from each fraction was corrected for background determinations of extracts incubated with *myo*-[^{3}H]Ins at time 0 h.

3.5. Incorporation of myo-[^{3}H]Ins into inositol phosphates

Procyclic forms expressing GPIPLC incorporated 28-fold more *myo*-[^{3}H]Ins into [^{3}H]IP, when stimulated with 2.5 μg ml^{-1} Tc for 24 h (Fig. 5 and Table 1). The difference in [^{3}H]IP$_3$ was just 1.5-fold, between parental and over-expressing cells. Non-induced *GPIPLC*-transformant procyclic cells showed a similar *myo*-[^{3}H]Ins incorporation as the parental procyclic cell line, and adding Tc to the parental cells did not change the rate of *myo*-[^{3}H]Ins incorporation into the different inositol phosphates (data not shown).

Uninduced *GPIPLC* conditional knock-out bloodstream forms showed 17-fold lower *myo*-[^{3}H]Ins incorporation into IP than the parental wild-type cell line (Table 1). Although independent experiments gave different levels of total incorporation, varying from 4 to 10% of the added *myo*-[^{3}H]Ins, shorter incorporation times, where the cells would be in better condition, showed similar results, although with lower amounts of total incorporation (data not shown). Irrespective of the total incorporation, the levels of [^{3}H]IP recovered from *GPIPLC* conditional knock-out bloodstream cells were an order of magnitude lower than those of wild-type cells. Tc induction consistently increased [^{3}H]IP about threefold, presumably by eliciting hydrolysis of PI. However, the amount of [^{3}H]IP was still fivefold lower than in the wild-type bloodstream-form cells. Adding Tc to the parental cell line did not modify the rate of *myo*-[^{3}H]Ins incorporation into inositol phosphates (data not shown). In both bloodstream and procyclic forms, the levels of [^{3}H]IP$_4$ were essentially unchanged by *GPIPLC* induction, further suggesting that GPIPLC is not involved in [^{3}H]IP$_4$ production.

These results suggest that GPIPLC can hydrolyze PI in bloodstream and procyclic forms, and that it can also hydrolyze PIP$_2$ to IP$_3$ in procyclic forms. Further experiments will be necessary to elucidate the details and the significance of these effects.

Table 1
Dependence of inositol phosphates levels on GPIPLC activity[a]

Clone	Life-cycle stage and GPIPLC status	IP	IP$_3$
17-19-18	Bloodstream knock-out uninduced	0.06 ± 0.02	0.9 ± 0.08
17-19-18	Bloodstream knock-out Tc-induced	0.18 ± 0.03	0.9 ± 0.2
29-13	Procyclic parental (wild-type)	6 ± 1.2	34 ± 3.6
29-13-19	Procyclic inducible GPIPLC Tc-induced	167 ± 11.5	50 ± 7.1

[a] [^{3}H]Ins labeling of inositol phosphates is expressed as a ratio to parental bloodstream-form control values. Comparison of *myo*-[^{3}H]inositol incorporation ratios between procyclic and bloodstream forms cannot be used to infer differences in absolute levels of IP and IP$_3$. Bloodstream forms were induced with 1 μg ml^{-1} Tc for 24 h and procyclic forms with 2.5 μg ml^{-1} Tc for 24 h.

4. Discussion

PI is required for the synthesis of glycolipid anchors in *T. brucei* (for a review, see [38], and for producing inositol phosphates and DAG [13]. In mammalian cells, DAG has been linked to PKC activation, which up-regulates cell growth and differentiation [17], and to DAG-kinase, which down-regulates the mitogenic signals that depend on nuclear DAG [39].

[^{3}H]inositol incorporation into IP and IP_3, in our experiments with wild-type bloodstream and procyclic cells, agreed well with the levels reported by other investigators [20]. Our observations suggest that *T. brucei* GPIPLC is able to cleave PI into IP and, presumably, DAG in vivo. The absence of GPIPLC depleted the production of [^{3}H]IP about 17-fold, compared with wild-type values. GPIPLC accounted for about 75% of wild-type bloodstream-form PI-hydrolyzing activity in vitro, which implies that *T. brucei* has alternative ways of making IP. The characterization of alternative PI-hydrolyzing enzymes may be important to understand the inositol phosphate pathways of *T. brucei*. Further investigation of these pathways was beyond our resources.

Ectopic expression of GPIPLC in procyclic cells also stimulated IP_3 production. In this respect, GPIPLC functioned like a PIP_2-specific higher eukaryotic PLCγ. The activity of PLCγ has been related to triggering growth and differentiation in several mammalian systems in response to many stimuli [21,40–46]. Activation of a wide variety of cell surface receptors, which in turn promote PIP_2 cleavage by PLCγ, ultimately releases Ca^{2+} in response to transient surges in IP_3, whose steady-state concentration is low, compared with the other inositol phosphates. PKC-like activities, sensitive to Ca^{2+} and DAG, have been described in *T. brucei* bloodstream and procyclic cells [47]. Despite the presence of IP_3 in wild-type procyclic cells, no IP_3-sensitive Ca^{2+} release could be detected in this life stage [20]. Thus, further studies on the ability of the GPIPLC-mediated IP_3 surge to elicit Ca^{2+} release will be necessary to comprehend the role of GPIPLC. The significance of such PIP_2-hydrolytic activity may illuminate the role of GPIPLC during different commitment points in the *T. brucei* life cycle.

If one wild-type bloodstream trypanosome contains about 30 000 molecules of GPIPLC [3], the mfVSG hydrolysis data from the *GPIPLC* knock-out cells indicated less than 10 molecules of GPI-PLC per trypanosome in the absence of Tc, about twice the assay blank value, and 16 000 molecules when the ectopic copy of *GPIPLC* was switched on. Wild-type and *GPIPLC*-expressing Tc-induced procyclic cells showed levels of mfVSG hydrolysis attributable to 45 and 29 000 molecules of GPIPLC per trypanosome, respectively. Thus, *GPIPLC* induction is tightly regulated, as originally shown for luciferase [27].

An unconditional GPIPLC null mutant was previously obtained in a less virulent trypanosome strain [7]. Why, therefore, were we unable to obtain a null mutant in *T. brucei* strain 427, despite several attempts? Infectivity, growth rate and morphology of strain 427 *GPIPLC* conditional knock-out bloodstream cultures were similar to wild-type cells, even under non-inducing conditions (data not shown). However, no clones could be recovered upon replacement of the *GPIPLC* second allele when Tc was absent from the selection medium. This suggests that very low levels of GPIPLC are critical for survival or rapid growth, in culture, of the 427 strain used in the present studies. This may be particularly true under the stressful conditions of transfection and cloning. The previously described null mutants were less virulent than the corresponding parental strain [7], which was already less virulent than strain 427. It is possible that, if true *T. brucei* strain 427 null mutants would have greatly reduced virulence, we might have been unable to select bloodstream-form null mutants directly. Bloodstream forms of the *T. brucei* strain used in the previous study could not be grown or transfected in vitro. Therefore, null mutants were made in the procyclic form, where GPIPLC is not normally expressed and may not be essential, and the bloodstream forms were derived by transmission through the Tsetse vector. This approach was not available to us.

T. brucei laboratory stocks often lose fly transmissibility after rapid passage through rodents,

together with a loss of trypanosome polymorphism in the mammalian host, and a reduced ability to differentiate in vitro [48]. The reasons for such changes remain elusive. However, the monomorphic 427 strain that was used in the current study can differentiate into procyclic forms in vitro, in the presence of citrate and/or *cis*-aconitate at 27°C [49,50], or in the Tsetse midgut. It can also complete its life cycle in the Tsetse, albeit with very low efficiency (unpublished data), and, under certain conditions, long-term cultured procyclic forms [51] or metacyclic forms produced by co-incubation of procyclic cells with Tsetse explants [52], can infect mice. Nevertheless, several differences between monomorphic and pleomorphic strains have been described upon differentiation, such as in protein synthesis [53] and faster loss of VSG and appearance of PARP in pleomorphic [54] than in monomorphic strains [50,55]. Several other biochemical pathways appear to differ between monomorphic and pleomorphic strains of *T. brucei*. Monomorphic strains show similar phosphoglycerate kinase activity as pleomorphic strains but higher levels of glycosomal phosphoglycerate kinase mRNA [56]. Differences in cAMP levels are also evident between monomorphic and pleomorphic strains during infection [57].

Our inability to obtain a null mutant in the 427 strain may be another example of differences between monomorphic and pleomorphic strains. In its potential role, regulating the pool of PIs involved in cellular growth, differentiation, and synthesis of glycolipid anchors, GPIPLC may be necessary for the survival or virulence of this strain. Further studies were beyond the scope of our current resources. The availability of a conditional GPIPLC knock-out in a virulent strain provides new opportunities for biochemical studies of PI and GPI pathways, which may identify the true function(s) of this enigmatic enzyme.

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References

[1] Cross GAM. Antigenic variation in trypanosomes: secrets surface slowly. Bioessays 1996;18:283–91.
[2] Fox JA, Duszenko M, Ferguson MAJ, Low MG, Cross GAM. Purification and characterization of a novel glycan-phosphatidylinositol-specific phospholipase C from *Trypanosoma brucei*. J Biol Chem 1986;261:15767–71.
[3] Bulow R, Overath P. Purification and characterization of the membrane-form variant surface glycoprotein hydrolase of *Trypanosoma brucei*. J Biol Chem 1986;261:11918–23.
[4] Hereld D, Krakow JL, Bangs JD, Hart GW, Englund PT. A phospholipase C from *Trypanosoma brucei* which selectively cleaves the glycolipid on the variant surface glycoprotein. J Biol Chem 1986;261:13813–9.
[5] Bulow R, Overath P. Synthesis of a hydrolase for the membrane-form variant surface glycoprotein is repressed during transformation of *Trypanosoma brucei*. FEBS Lett 1985;187:105–10.
[6] Carrington M, Bulow R, Reinke H, Overath P. Sequence and expression of the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. Mol Biochem Parasitol 1989;33:289–96.
[7] Webb H, Carnall N, Vanhamme L, Rolin S, van den Abbeele J, Welburn S, Pays E, Carrington M. The GPI-phospholipase C of *Trypanosoma brucei* is nonessential but influences parasitemia in mice. J Cell Biol 1997;139:103–14.
[8] Rolin S, Hancocq-Quertier J, Paturiaux-Hanocq F, Nolan DP, Pays E. Mild acid stress as a differentiation trigger in *Trypanosoma brucei*. Mol Biochem Parasitol 1998;93:251–62.
[9] Bulow R, Nonnengasser C, Overath P. Release of the variant surface glycoprotein during differentiation of bloodstream to procyclic forms of *Trypanosoma brucei*. Mol Biochem Parasitol 1989;32:85–92.
[10] Ziegelbauer K, Stahl B, Karas M, Stierhof YD, Overath P. Proteolytic release of cell surface proteins during differentiation of *Trypanosoma brucei*. Biochemistry 1993;32:3737–42.
[11] Mutomba MC, Wang CC. The role of proteolysis during differentiation of *Trypanosoma brucei* from the bloodstream to the procyclic form. Mol Biochem Parasitol 1998;93:11–22.
[12] Bulow R, Griffiths G, Webster P, Stierhof Y-D, Opperdoes FR, Overath P. Intracellular localization of the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. J Cell Sci 1989;93:233–40.

[13] Butikofer P, Boschung M, Brodbeck U, Menon AK. Phosphatidylinositol hydrolysis by *Trypanosoma brucei* glycosylphosphatidylinositol phospholipase c. J Biol Chem 1996;271:15533–41.
[14] Kuppe A, Evans LM, McMillen DA, Griffith OH. Phosphatidylinositol-specific phospholipase C of Bacillus cereus: cloning, sequencing, and relationship to other phospholipases. J Bacteriol 1989;171:6077–83.
[15] Leimeister-Wachter M, Domann E, Chakraborty T. Detection of a gene encoding a phosphatidylinositol-specific phospholipase C that is co-ordinately expressed with listeriolysin in Listeria monocytogenes. Mol Microbiol 1991;5:361–6.
[16] Dawson AP. Calcium signalling: how do IP3 receptors work? Curr Biol 1997;7:544–7.
[17] Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 1995;9:484–96.
[18] Guther ML, Treumann A, Ferguson MA. Molecular species analysis and quantification of the glycosylphosphatidylinositol intermediate glycolipid C from *Trypanosoma brucei*. Mol Biochem Parasitol 1996;77:137–45.
[19] Daniels CJ, Palmer FB. Biosynthesis of phosphatidylinositol in *Crithidia fasciculata*. Biochim Biophys Acta 1980;618:263–81.
[20] Moreno SN, Docampo R, Vercesi AE. Calcium homeostasis in procyclic and bloodstream forms of *Trypanosoma brucei*. Lack of inositol 1,4,5-trisphosphate-sensitive Ca2 + release. J Biol Chem 1992;267:6020–6.
[21] Wang Z, Gluck S, Zhang L, Moran MF. Requirement for phospholipase C-gamma1 enzymatic activity in growth factor-induced mitogenesis. Mol Cell Biol 1998;18:590–7.
[22] Fernandez S, Homann MJ, Henry SA, Carman GM. Metabolism of the phospholipid precursor inositol and its relationship to growth and viability in the natural auxotroph *Schizosaccharomyces pombe*. J Bacteriol 1986;166:779–86.
[23] Brodbeck U, Gentinetta R, Ott P. In: Azzi A, Brodbeck U, Zahler P, editors. Membrane proteins: a laboratory manual. Berlin: Springer-Verlag, 1981:85–96.
[24] Cross GAM. Identification, purification and properties of variant-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. Parasitology 1975;71:393–417.
[25] Hirumi H, Hirumi K. Continuous cultivation of *Trypanosoma brucei* bloodstream forms in a medium containing a low concentration of serum protein without feeder cell layers. J Parasitol 1989;75:985–9.
[26] Brun R, Schonenberger. Cultivation and in vitro cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop 1979;36:289–292.
[27] Wirtz E, Leal S, Ochatt C, Cross GAM. A tightly regulated inducible expression system for dominant negative approaches in *Trypanosoma brucei*. Mol Biochem Parasitol 1999;99:91–103.
[28] Clayton C, Adams M, Almeida R, Baltz T, Barrett M, Bastien P, Belli S, Beverley S, Biteau N, Blackwell J, Blaineau C, Boshart M, Bringaud F, Cross G, Cruz A, Degrave W, Donelson J, El-Sayed N, Fu GL, Ersfeld K, Gibson W, Gull K, Ivens A, Kelly J, Lawson D. Genetic nomenclature for *Trypanosoma* and *Leishmania*. Mol Biochem Parasitol 1998;97:221–4.
[29] Carruthers VB, van der Ploeg LH, Cross GA. DNA-mediated transformation of bloodstream-form *Trypanosoma brucei*. Nucl Acids Res 1993;21:2537–8.
[30] Navarro M, Cross GAM. DNA rearrangements associated with multiple consecutive directed antigenic switches in *Trypanosoma brucei*. Mol Cell Biol 1996;16:3615–25.
[31] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY, 1989.
[32] Bordier C. Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem 1981;256:1604–7.
[33] Ellman GL, Courtney DK, Andres VJ, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
[34] Morris JC, Pingsheng L, Zhai HX, Shen TY, Mensa-Wilmot K. Phosphatidylinositol phospholipase C is activated allosterically by the aminoglycoside G418-2-deoxy-2-fluoro-scyllo-inositol-1-o-dodecylphosphonate and its analogs inhibit glycosylphosphatidylinositol phospholipase C. J Biol Chem 1996;271:15468–77.
[35] Berridge MJ, Dawson RM, Downes CP, Heslop JP, Irvine RF. Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem J 1983;212:473–82.
[36] Cusack B, Groshan K, McCormick D, Pang Y-P, Perry R, Phung C-T, Souder T, Richelson E. Chimeric rat/human neurotensin receptors localize a region of the receptor sensitive to binding of a novel, species-specific, picomolar affinity peptide. J Biol Chem 1996;271:15045–59.
[37] Armah D, Mensa-Wilmot K. Myristoylation of GPIPLC in *T. brucei* influences activity. J Biol Chem 1999;274:5931–8.
[38] Ferguson MA. Colworth Medal Lecture. Glycosyl-phosphatidylinositol membrane anchors: the tale of a tail. Biochem Soc Trans 1992;20:243–56.
[39] Tophan MK, Bunting M, Zimmerman GA, McIntyre TM, Blackshear PJ, Prescott SM. Protein Kinase C regulates the nuclear localization of siacylglycerol kinase. Nature 1998;394:697–700.
[40] Facchinetti MM, Boland R, de Boland AR. Age-related loss of calcitriol stimulation of phosphoinositide hydrolysis in rat skeletal muscle. Mol Cell Endocrinol 1998;136:131–8.
[41] Goppelt-Struebe M, Stroebel M. Signaling pathways mediating induction of the early response genes prostaglandin G/H synthase-2 and egr-1 by serotonin via 5-HT2A receptors. J Cell Physiol 1998;175:341–7.
[42] Ji QS, Ermini S, Baulida J, Sun FL, Carpenter G. Epidermal growth factor signaling and mitogenesis in Plcg1 null mouse embryonic fibroblasts. Mol Biol Cell 1998;9:749–57.

[43] Melien O, Thoresen GH, Sandnes D, Ostby E, Christoffersen T. Activation of p42/p44 mitogen-activated protein kinase by angiotensin II, vasopressin, norepinephrine, and prostaglandin F2alpha in hepatocytes is sustained, and like the effect of epidermal growth factor, mediated through pertussis toxin-sensitive mechanisms. J Cell Physiol 1998;175:348–58.

[44] Sauer H, Hofmann C, Wartenberg M, Wobus AM, Hescheler J. Spontaneous calcium oscillations in embryonic stem cell-derived primitive endodermal cells. Exp Cell Res 1998;238:13–22.

[45] Xie H, Pallero MA, Gupta K, Chang P, Ware MF, Witke W, Kwiatkowski DJ, Lauffenburger DA, Murphy-Ullrich JE, Wells A. EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLCgamma signaling pathway. J Cell Sci 1998;111:615–24.

[46] Scharenberg AM, Kinet J-P. PtdIns-3,4,5-P3: a regulatory nexus between tyrosine kinases and sustained Calcium signals. Cell 1998;94:5–8.

[47] Keith K, Hide G, Tait A. Characterisation of protein kinase C like activities in *Trypanosoma brucei*. Mol Biochem Parasitol 1990;43:107–16.

[48] Hajduk SL, Vickerman K. Absence of detectable alteration in the kinetoplast DNA of a *Trypanosoma brucei* clone following loss of ability to infect the insect vector (*Glossina morsitans*). Mol Biochem Parasitol 1981;4:17–28.

[49] Czichos J, Nonnengaesser C, Overath P. *Trypanosoma brucei*: cis-aconitate and temperature reduction as triggers of synchronous transformation of bloodstream to procyclic trypomastigotes in vitro. Exp Parasitol 1986;62:283–91.

[50] Roditi I, Schwarz H, Pearson TW, Beecroft RP, Liu MK, Williams RO, Overath P. Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of *Trypanosoma brucei*. J Cell Biol 1989;108:737–46.

[51] Cross GAM. Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. Parasitology 1975;71:393–417.

[52] Cunningham I, Honigberg BM, Taylor AM. Infectivity of monomorphic and pleomorphic *Trypanosoma brucei* stocks cultivated at 28 C with various tsetse fly tissues. J Parasitol 1981;67:391–7.

[53] Bass KE, Wang CC. Transient inhibition of protein synthesis accompanies differentiation of *Trypanosoma brucei* from bloodstream to procyclic forms. Mol Biochem Parasitol 1992;56:129–40.

[54] Pays E, Hanocq-Quertier J, Hanocq F, van Assel S, Nolan D, Rolin S. Abrupt RNA changes precede the first cell division during the differentiation of *Trypanosoma brucei* bloodstream forms into procyclic forms in vitro. Mol Biochem Parasitol 1993;61:107–14.

[55] Mutomba MC, Wang CC. Differentiation of a culture-adapted mutant bloodstream form of *Trypanosoma brucei* into the procyclic form results in growth arrest of the cells. Mol Biochem Parasitol 1995;72:215–25.

[56] Parsons M, Hill T. Elevated phosphoglycerate kinase mRNA but not protein in monomorphic *Trypanosoma brucei*: implications for stage-regulation and post- transcriptional control. Mol Biochem Parasitol 1989;33:215–27.

[57] Mancini PE, Patton CL. Cyclic 3′,5′-adenosine monophosphate levels during the developmental cycle of *Trypanosoma brucei* brucei in the rat. Mol Biochem Parasitol 1981;3:19–31.