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Phosphatidylinositol synthesis is essential in bloodstream form *Trypanosoma brucei*

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PI (phosphatidylinositol) is a ubiquitous eukaryotic phospholipid which serves as a precursor for messenger molecules and GPI (glycosylphosphatidylinositol) anchors. PI is synthesized either *de novo* or by head group exchange by a PIS (PI synthase). The synthesis of GPI anchors has previously been validated both genetically and chemically as a drug target in *Trypanosoma brucei*, the causative parasite of African sleeping sickness. However, nothing is known about the synthesis of PI in this organism. Database mining revealed a putative *TbPIS* gene in the *T. brucei* genome and by recombinant expression and characterization it was shown to encode a catalytically active PIS, with a high specificity for *myo*-inositol. Immunofluorescence revealed that in *T. brucei*, PIS is found in both the endoplasmic reticulum and Golgi. We created a conditional double knockout of *TbPIS* in the bloodstream form of *T. brucei*, which when grown under non-permissive conditions, clearly showed that *TbPIS* is an essential gene. *In vivo* labelling of these conditional double knockout cells confirmed this result, showing a decrease in the amount of PI formed by the cells when grown under non-permissive conditions. Furthermore, quantitative and qualitative analysis by GLC-MS and ESI-MS/MS (electrospray ionization MS/MS) respectively showed a significant decrease (70 %) in cellular PI, which appears to affect all major PI species equally. A consequence of this fall in PI level is a knock-on reduction in GPI biosynthesis which is essential for the parasite's survival. The results presented here show that PI synthesis is essential for bloodstream form *T. brucei*, and to our knowledge this is the first report of the dependence on PI synthesis of a protozoan parasite by genetic validation.

Key words: bloodstream form, essentiality, glycosylphosphatidylinositol, *myo*-inositol, phosphatidylinositol synthase (PIS), *Trypanosoma*.

INTRODUCTION

In eukaryotes, PI (phosphatidylinositol) is a ubiquitous phospholipid that forms between 3 and 10 % of cell membranes, functions as a precursor for cell signalling molecules and provides the basic building block used in GPI (glycosylphosphatidylinositol) anchor biosynthesis. PI is synthesized *de novo* via the action of a PIS (PI synthase; EC 2.7.8.11) using *myo*-inositol and CDP-DAG (CDP diacylglycerol) and releasing CMP. Alternatively, in the absence of CDP-DAG, the head group may be exchanged between pre-existing PI and free *myo*-inositol. Native and recombinant PIS enzymes have been studied from many organisms including *Saccharomyces cerevisiae* [1–4], *Arabidopsis thaliana* [5–7] and *Toxoplasma gondii* [8]. PIS enzymes appear to be predominantly localized to the ER (endoplasmic reticulum), although they have also been detected in other cellular locations such as Golgi [9], outer mitochondrial membrane in *S. cerevisiae* [1,4] and plasma membrane in rat pituitary GH_3 cells [10]. To date, all PIS enzymes require Mg^{2+} or Mn^{2+} for activity and have neutral pH optima. Although the ability to catalyse both the PI synthesis and exchange reactions has not been investigated for all PIS enzymes, it has been clearly shown for recombinant PISs from several organisms, in particular *S. cerevisiae* [2] and *A. thaliana* [6]. However, the exact mechanism for this reaction and its physiological significance remain unknown.

African trypanosomiasis is caused by the protozoan parasite *Trypanosoma brucei* and is both a potentially fatal disease and a serious economic problem in sub-Saharan Africa. This unicellular parasite is able to avoid the host's innate immune system by undergoing antigenic variation that involves switching of GPI-anchored VSGs (variant-surface glycoproteins) [11]. Despite the variation of the VSG protein, the GPI core structure attached to protein remains unchanged and comprises $NH_2CH_2CH_2PO_4$H-6Manα1-2Manα1-6Manα1-4GlcNα1-6D-*myo*-inositol-1-HPO_4-dimyristylglycerol [12]. The biosynthesis of GPI anchors has been both genetically and chemically validated as a potential therapeutic drug target in bloodstream form *T. brucei* [13–15].

PI is utilized in the initial step of GPI anchor biosynthesis, where GlcNAc is transferred from UDP-GlcNAc to PI to form GlcNAc-PI (see [16] and references contained therein). Surprisingly, despite the essentiality of GPI anchors to bloodstream form *T. brucei*, very little is known about PI biosynthesis in these parasites. The synthesis of PI has been demonstrated in *Plasmodium knowlesi* [17], *Plasmodium falciparum* [17], *Crithidia fasciculate* [18] and *Giardi lamblia* [19], although to date PIS synthesis has not been shown to be essential for the survival of these parasites. The only report of molecular cloning and characterization of a protozoan PIS is from *To. gondii*, in which two developmentally regulated *PIS* genes have been identified [8].

Abbreviations used: BiP, endoplasmic reticulum luminal chaperone binding protein; DAG, diacylglycerol; DAPI, 4,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; ESI-MS, electrospray ionization MS; GPI, glycosylphosphatidylinositol; HA, haemagglutinin; HPTLC, high-performance TLC; HYG, hygromycin phosphotransferase; IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani; Ni-NTA, Ni^{2+}-nitrilotriacetate; ORF, open reading frame; PAC, puromycin acetyltransferase; PI, phosphatidylinositol; PLC, phospholipase C; PI-PLC, PI-specific PLC; PIS, PI synthase; TbGRASP, *T. brucei* Golgi matrix protein; TbPIS, *T. brucei* PI synthase; TDB, trypanosome dilution buffer; Ti, tetracycline-inducible; TRITC, tetramethylrhodamine β-isothiocyanate; UTR, untranslated region; VSG, variant-surface glycoprotein.

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The nucleotide sequence reported in this paper has been submitted to DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AJ716153.

In the present study, we report investigations into PI synthesis in bloodstream form *T. brucei*, involving molecular cloning of the PIS, recombinant expression and preliminary enzyme characterization. We also show, through creation of a conditional double knockout, that PI synthesis is essential to the survival of the bloodstream form of the parasite. To our knowledge, this is the first report of essentiality of PI synthesis in any protozoan parasite.

EXPERIMENTAL

Nucleic acid manipulations

Using the *S. cerevisiae* PIS, a putative *PIS* gene was identified in the *T. brucei* genome database (Sanger Centre, Cambridge, U.K.) using tBlastN. The ORF (open reading frame) was PCR-amplified from *T. brucei* genomic DNA with Pfu polymerase using the forward and reverse primers 5′-GAGGAGAAGCTTATGCCGAAAGCTAAAACT-3′ and 5′-TCGTTAATTAACTGGCGGCTTCCCGCAGC-3′ respectively. The amplicon was purified (QIAquick PCR purification kit; Qiagen), cloned into pCR-Blunt II TOPO (Invitrogen) and sequenced. Using the HindIII and PacI restriction sites (underlined in primer sequences), the putative *TbPIS* (*T. brucei* PIS gene) was ligated into the tetracycline-inducible expression vectors pLew82 and pLew100 [20] via the HindIII and PacI restriction sites.

To construct the *T. brucei* gene replacement cassettes, the 5′-UTR (5′-untranslated region) and 3′-UTR immediately adjacent to the *PIS* ORF were amplified from *T. brucei* genomic DNA using Pfu polymerase. The primers 5′-ATAAGAATGCGGCCGCATAATCACTTTAGCGTCGCGTGG-3′ and 5′-GTTTAAACTTACGGACCGTCA*AGCTT*TGGTGCTGCGTTGCTTGC-3′ were used for the 5′-UTR, and primers 5′-GACGGTCCGTAAGTTTAAACG*GATCC*GGAGTTGTGTGTTAAAGG-3′ and 5′-ATAAGAATGCGGCCGCATTCCACACCAATAAAAGGAGAT-3′ for the 3′-UTR. These amplified products were used in a knitting PCR, in which the 5′-UTR was joined to the 3′-UTR via a short BamHI–HindIII linker region contained within the described primers (italics) and a NotI site (underlined) at each end. This PCR product was ligated into pGEM-5Zf(+) (Promega) via the NotI sites and the hygromycin [HYG (hygromycin phosphotransferase)] or puromycin [PAC (puromycin acetyltransferase)] resistance genes were ligated between the BamHI and HindIII restriction sites. Plasmid DNA was prepared using a QIAprep Miniprep Plasmid kit (Qiagen); after digestion with NotI, it was precipitated with sodium acetate/ethanol and dissolved in sterile water to a final concentration of 1 μg/μl for electroporation.

Southern and Northern blots

The *PIS* ORF was PCR-amplified using the same primers described in the previous section for ligation into pLew vectors and gel-purified with a QIAquick gel extraction kit (Qiagen). This fragment was then labelled with either fluorescein (Gene Images-Random prime module; Amersham) for Southern blotting or [α-^{32}P]dCTP (RediprimeII random prime labelling system; Amersham) for Northern blotting.

For Southern blots, genomic *T. brucei* DNA (2 μg) was digested with various restriction enzymes, the digestion products were separated on a 0.8 % agarose gel and transferred on to a Hybond-N membrane (Amersham). The membrane was hybridized overnight in ULTRA-HYB (Ambion) at 42 °C with the fluorescein-labelled *PIS* ORF probe. Stringency washes were carried out at 42 °C, and consisted of two washes at low stringency for 15 min each (2 × SSC and 0.1 % SDS; 1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and two washes at high stringency again for 15 min each (0.2 × SSC and 0.1 % SDS). Bound probe was detected using a CDP-STAR detection module (Amersham) and autoradiography.

For Northern blots, total RNA was purified using an RNeasy Mini kit (Qiagen) and separated on a formaldehyde gel and transferred on to Hybond N+ (Amersham). Hybridization conditions and stringency washes were as described for Southern blotting, using the ^{32}P-labelled *PIS* ORF as the probe. Bound probe was detected by autoradiography.

Cultivation and genetic modification of *T. brucei*

Bloodstream form *T. brucei* strain 427, which has been previously modified to express both T7 polymerase and the tetracycline repressor protein [20], is referred to here as wild-type cells for convenience. Cells were grown in HMI-9 medium supplemented with G418 (2.5 μg/ml), at 37 °C with 5 % CO_2 as described elsewhere [13,20–22]. Transformation conditions and subsequent drug selection were also described elsewhere [13,20–22]. For experiments requiring tetracycline-free conditions, Tet-system-approved foetal calf serum (Clontech) was used. When tetracycline was added to the medium, a final concentration of 1 μg/ml was used.

Immunofluorescence

Mid-exponential cells were collected by centrifugation (800 ***g***, 10 min) and fixed with 4 % (w/v) paraformaldehyde. After washing with PBS, the fixed cells were allowed to adhere to poly-lysine slides prior to staining with either DAPI (4,6-diamidino-2-phenylindole; 2 μg/ml) or antibodies. Cells were rehydrated with PBS and washed with PBS–glycine (0.1 M) prior to permeabilization with Triton X-100 (0.1 %) and blocking with 1 % BSA in PBS. To detect the HA (haemagglutinin) tag, cells were incubated with the rat monoclonal anti-HA antibody (Roche) and FITC-conjugated rabbit anti-rat immunoglobulins (DakoCytomation). ER and Golgi localization was determined by incubation for 1 h at room temperature (~20 °C) with either rabbit anti-BiP (ER luminal chaperone binding protein) [23] or rabbit anti-TbGRASP (*T. brucei* Golgi matrix protein) antibodies [24] respectively, prior to incubation for 1 h at room temperature with TRITC (tetramethylrhodamine β-isothiocyanate)-conjugated anti-rabbit immunoglobulins (Sigma).

Recombinant expression in *Escherichia coli*

TbPIS was amplified from genomic DNA using the primers 5′-GGCGATATCGGATCCCATGCCGAAAGCTAAAACT-3′ and 5′-CCGCAAGCTTGGGCTGGCGGCTTCCCGCAGCAGCATCCA-3′ and ligated into pET32b (Novagen) via the sites BamHI and HindIII, which are underlined in the primer sequences. For recombinant expression, freshly transformed BL21 cells were used to inoculate 100 ml of M9 glucose minimal medium (M9 salts, 0.2 % glucose, 1 mM $MgSO_4$ and 0.001 % thiamine) supplemented with casamino acids (2 g/l) and ampicillin (100 μg/ml). Cells were grown at 37 °C until the absorbance (*A*) at 600 nm was between 0.5 and 0.6, then induced with IPTG (isopropyl β-D-thiogalactoside; 50 μM) and grown overnight at 25 °C. The cells were collected by centrifugation and resuspended in lysis buffer [50 mM sodium phosphate, pH 7, 300 mM NaCl, 5 mM 2-mercaptoethanol, 5 % (v/v) glycerol, 0.1 % Triton X-100, 40 mM imidazole and complete protease inhibitor cocktail without EDTA (Roche)], before disruption by sonication. After the supernatant was cleared by centrifugation, prewashed Ni-NTA (Ni^{2+}-nitrilotriacetate) beads (Qiagen) were added and the mixture was incubated at 4 °C for 1 h. The beads were collected,

washed with lysis buffer and bound protein was eluted with lysis buffer containing 250 mM imidazole, and detected by Western blotting using anti-His antibodies (Clontech).

myo-[^{3}H]Inositol labelling of *E. coli* cultures

In vivo labelling of *E. coli* cells expressing TbPIS was performed as described previously [5]. Briefly, cells were grown in 10 ml of LB (Luria–Bertani) medium supplemented with 0.1 mM *myo*-inositol until the A_{600} was 0.8, and then 4 ml of culture was removed, washed in LB medium and resuspended in 2 ml of LB medium supplemented with 20 μM *myo*-inositol and 1.2 μM [^{3}H]*myo*-inositol. Expression was induced with 0.5 mM IPTG and the cells were grown at 37 °C for a further 3 h. The cells were collected by centrifugation, resuspended in sonication buffer (50 mM Tris/HCl, pH 8, 2 mM EDTA and 0.2 mg/ml lysozyme) and disrupted by sonication, and whole cells and debris were removed by centrifugation (4500 ***g***, 10 min). To extract lipids, chloroform and methanol were added to the supernatant to achieve a final concentration of 10:10:3 (by vol.) chloroform/methanol/water and incubated with shaking at room temperature for 1 h, and samples were then nitrogen dried and desalted by butanol/water partitioning. Lipids were separated by HPTLC (high-performance TLC) using silica 60 plates with chloroform/methanol/water (10:10:3) as the solvent. Radiolabelled lipids were detected by fluorography at −70 °C after spraying with En^{3}Hance™ (NEN) and using a Kodak XAR-5 film with an intensifying screen.

E. coli membrane preparation and PIS enzyme assay

For *E. coli* membrane purification, BL21 cells freshly transformed with the *TbPIS*-pET32b vector were induced and grown overnight as described above. After collection by centrifugation, cells were resuspended in PBS and disrupted by sonication. Cell debris, whole cells and ghosts were removed by slow-speed centrifugation (14 500 ***g***, 10 min); subsequently, membranes were collected by high-speed centrifugation (100 000 ***g***, 1 h), resuspended in 50 mM Tris (pH 8), 5 mM EDTA and 20 % glycerol and stored as aliquots at −20 °C.

Unless otherwise stated, the reaction mixture for PIS activity consisted of 50 mM Tris/HCl (pH 8), 0.15 mM CDP-DAG (Avanti Polar Lipids), 0.3 % n-octyl glucopyranoside, 2 μCi of [^{3}H]*myo*-inositol (Amersham; 14 Ci/mmol), 2.5 mM $MnCl_2$, 20 mM $MgCl_2$ and 50 μg of membrane protein, in a final volume of 100 μl. These reaction conditions were used to investigate substrate recognition with various isomers and analogues of inositol at 25 μM and 1 mM. Reaction mixtures were incubated at 30 °C for 1 h and terminated by the addition of 666 μl of chloroform/methanol (1:1, v/v). Lipid extractions and separations were performed as described for [^{3}H]*myo*-inositol labelling of *E. coli* cells. [^{3}H]PI was quantified using an AR-2000 imaging scanner (Bioscan).

The reaction mixture used to investigate the exchange reaction was based on that of Klezovitch et al. [2] and contained 50–100 μg of membrane protein, 0.5 μM PI (soya bean; Sigma), 2 μCi of [^{3}H]*myo*-inositol, 25 mM $MnCl_2$, 50 mM $MgCl_2$ and 100 mM Tris/HCl (pH 8) either with or without CMP (4 mM).

In vivo T. brucei metabolic labelling

All results presented for *in vivo* metabolic labelling are representative of three independent experiments and all errors were within 5 %. For metabolic labelling, 2×10^7 mid-exponential cells (1×10^6 cells/ml) were centrifuged (800 ***g***, 10 min), washed in inositol-free minimal essential medium [15,25,26], before being resuspended in the same medium at a final concentration of 1×10^7 cells/ml. Cells were labelled for 1 h at 37 °C with 50 μCi/ml of either D-[2-^{3}H]inositol (30 Ci/mmol; Amersham) or D-[2-^{3}H]mannose (14 Ci/mmol; Amersham). The cells were collected by centrifugation (800 ***g***, 10 min) and lipids were extracted in chloroform/methanol/water (10:10:3) for 1 h, the supernatant was removed and the pellet was re-extracted with chloroform/methanol (2:1) for 1 h. The supernatants were pooled and dried under a stream of nitrogen prior to desalting using butanol/water partitioning. An aliquot of this lipid fraction was taken and the total ^{3}H c.p.m. in this lipid fraction was determined by scintillation spectrometry using a Beckman LS6000SE with Formula 989 scintillation fluid (Packard Bioscience). Lipids were separated by HPTLC using silica 60 HPTLC plates with chloroform/methanol/water (10:10:3) as the solvent. Radiolabelled lipids were detected by fluorography at −70 °C, after spraying with En^{3}Hance™ and using a Kodak XAR-5 film with an intensifying screen. [^{3}H]PI was quantified using an AR-2000 imaging scanner (Bioscan).

When labelling with [^{35}S]methionine, 1×10^7 mid-exponential cells were collected by centrifugation, washed in methionine-free minimal essential media and resuspended in the same medium at a final concentration of 1×10^7 cells/ml. The cells were labelled for 30 min with 20 μCi [^{35}S]methionine (1175 Ci/mmol; MP Biomedicals) at 37 °C. To quench the labelling, the cells were diluted in 20 ml of cold TDB (trypanosome dilution buffer; 25 mM KCl, 400 mM NaCl, 5 mM $MgSO_4$, 100 mM Na_2HPO_4, NaH_2PO_4 and 100 mM glucose) containing 1 mM L-methionine and centrifuged (800 ***g***, 10 min, 4 °C). After the supernatant was removed, the cells were resuspended in TDB, an equal volume of 2× SDS/PAGE sample buffer (100 mM Tris, pH 6.8, 2 % SDS, 0.1 % Bromophenol Blue, 10 % glycerol and 5 % 2-mercaptoethanol) was added and heated at 100 °C. Proteins were separated on an SDS/10 % polyacrylamide gel and visualized by Coomassie Blue staining. To detect ^{35}S-labelled proteins, destained gel was soaked in En^{3}Hance™ for 30 min, washed with water twice, soaked in 10 % glycerol and dried. The dried gel was then exposed to an XAR-5 film overnight at −70 °C.

Enzymatic digests and chemical characterization of radiolabelled lipid species

Digestion with PI-PLC [PI-specific PLC (phospholipase C); Glyko], deamination, base hydrolysis and HF (hydrogen fluoride) treatment were as described previously [25–27].

Inositol analysis

Mid-exponential cells were collected by centrifugation (800 ***g***, 10 min), washed with TDB and stored at −20 °C. Lipids were extracted from these samples by the addition of 500 μl of chloroform/methanol mixture (2:1) and incubated at room temperature for 1 h. The supernatant was removed and the pellet was re-extracted with chloroform/methanol/water mixture (1:2:0.8). The supernatants were pooled and dried under nitrogen prior to desalting by biphasic partitioning using 2:1 (v/v) butanol/water. An internal standard of D_6 *myo*-inositol was added to samples prior to hydrolysis by strong acid (6 M HCl, 110 °C), derivitization with TMS [trimethylsilyl (Me_3Si)] and analysis by GLC-MS, by the method of Ferguson [28]. *myo*-Inositol was quantified and the means for three separate analyses were determined.

ESI-MS

Mid-exponential cells were collected by centrifugation (800 ***g***, 10 min), washed once with PBS and resuspended in PBS. Lipids

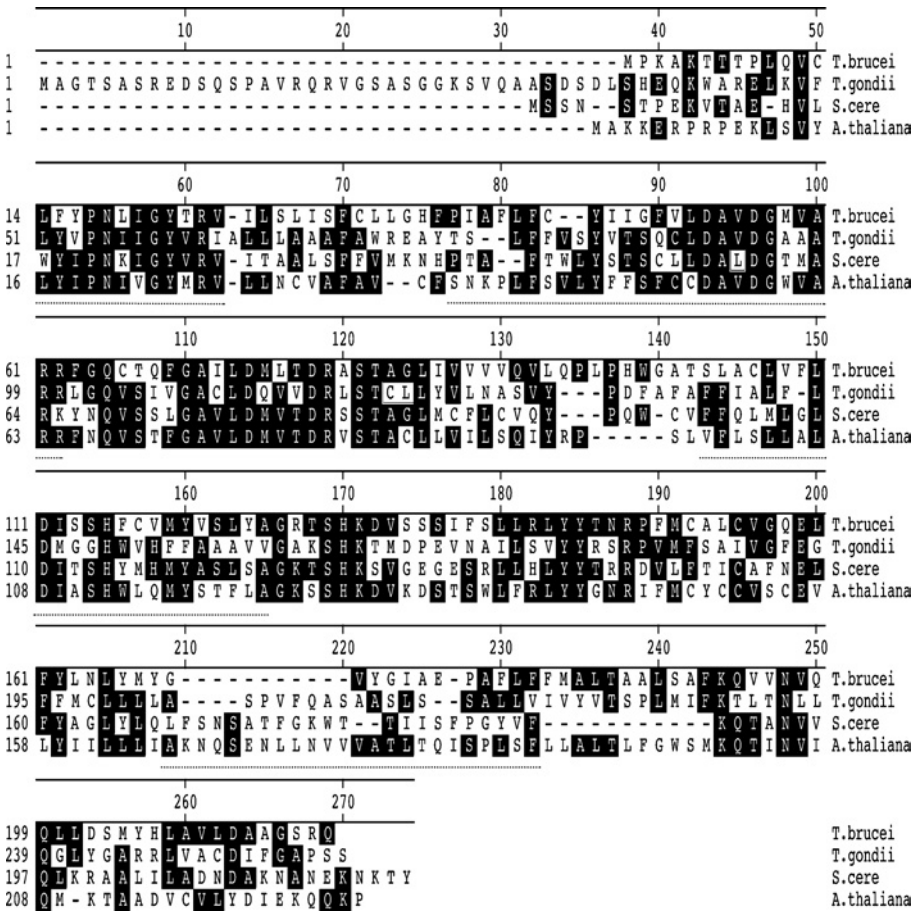

Figure 1 Clustal W alignment of the predicted amino acid sequence of the TbPIS (AJ716153) with those of *S. cerevisiae* (S. cere, GenBank® accession no. J02697), *To. gondii* (AF069132) and *A. thaliana* (AJ000539)

Identical residues are shown in reverse type with black background. Predicted transmembrane domains of the TbPIS are underlined.

were extracted by the method of Bligh and Dyer [29]. Qualitative analysis of lipids was performed by ESI-MS (electrospray ionization MS) and ESI-MS/MS with nanospray tips (Micromass type F) using a Micromass Quattro Ultima triple quadrupole mass spectrometer with argon [3.0×10^{-3} Torr (1 Torr = 133.3 Pa)] as the collision gas. A capillary voltage of 0.9 kV was used for both positive- and negative-ion modes. Cone voltages of 50 and 30 V were used for positive- and negative-ion modes respecttively. For analysis of inositol-phospholipids, negative-ion parent ion scanning of *m*/*z* 241 (corresponding to the collision-induced fragment inositol-1,2-cyclic phosphate) was used with collision energy of 45 V.

RESULTS AND DISCUSSION

Cloning of *TbPIS*

A putative PIS was identified in the *T. brucei* genome database using the *S. cerevisiae* PIS as the query. This putative *TbPIS* ORF was amplified and the sequence was confirmed. The predicted molecular mass of the deduced amino acid sequence is 24 kDa; an alignment of the deduced amino acid sequence with deduced PIS proteins from other organisms is shown in Figure 1. As with all other PISs (e.g. 1, 5 and 8), the TbPIS contains four putative transmembrane domains (underlined in Figure 1) as predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html). The putative TbPIS also contains a perfect copy of the motif [$DG(X)_2AR(X)_8G(X)_3D(X)_3D$] between residues 55 and 78, which has been described in all other phospholipid-synthesizing enzymes identified to date. The *TbPIS* has been submitted to GenBank® Nucleotide Sequence Database under the accession number AJ716153.

Recombinant expression

To confirm the enzymatic activity of the putative TbPIS, it was expressed in *E. coli* using the pET32b vector that encodes both N- and C-terminal His_6 tags. The production of a His-tagged protein of the correct molecular mass (38 kDa) was confirmed by Western blotting with detection by anti-His antibodies (Figure 2A).

As *E. coli* do not possess endogenous PIS activity, the recombinant TbPIS activity was confirmed by two methods. Firstly, when *in vivo* [^{3}H]*myo*-inositol labelling of the *E. coli* cells expressing the TbPIS (Figure 2B) was undertaken, a [^{3}H]*myo*-inositol lipid species was only present in cell extracts from *E. coli* cells expressing TbPIS (Figure 2B, lane 1). This [^{3}H]*myo*-inositol lipid species had an R_f (retention factor) identical with that of PI; further characterization showed it to be sensitive to PI-PLC, base hydrolysis and HF treatment (results not shown). These results are consistent with the [^{3}H]*myo*-inositol species being PI and clearly show that the putative TbPIS is a catalytically active PIS.

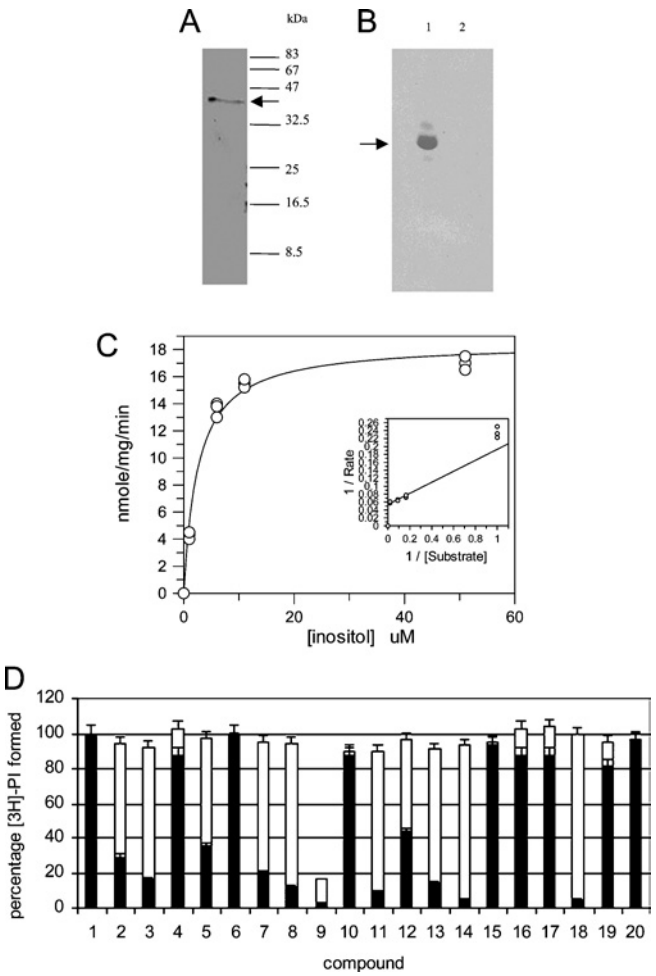

Figure 2 Expression of TbPIS in *E. coli* and some biochemical characterization

(**A**) *TbPIS* was cloned into the expression vector pET32b and expressed in BL21 *E. coli* cells. Recombinant protein was purified from lysed cells in the presence of detergent using Ni-NTA beads. Purified protein was run on an SDS/10% polyacrylamide gel, transferred on to nylon membrane by Western blotting and detected using anti-His antibodies. Arrow indicates purified His-tagged TbPIS. (**B**) *E. coli* cells expressing TbPIS (lane 1) or unrelated protein (lane 2) were labelled *in vivo* with [^{3}H]*myo*-inositol, and lipids were extracted and separated by HPTLC as described in the text. [^{3}H]*myo*-Inositol-labelled lipids were detected by autoradiography; arrow indicates [^{3}H]*myo*-inositol-labelled PI. (**C**) Kinetics of recombinant TbPIS for *myo*-inositol. Enzyme activity was measured as described in the Experimental section; CDP-DAG concentration was held constant (0.15 mM) and inositol concentration varied. Inset shows a Lineweaver–Burk plot of the data. (**D**) Ability of TbPIS to utilize inositol isomers or chemically modified inositol to form PI. Compound 1, *muco*-; compound 2, *neo*-; compound 3, *epi*-; compound 4, D(+)-*chiro*-; compound 5, L(−)-*chiro*; compound 6, *cis*-; compound 7, *allo*-; compound 8, *scyllo*-; compound 9, *myo*-; compound 10, 2-OMe-*chiro*-; compound 11, 1-deoxy-1-fluoro-*scyllo*-; compound 12, 2-OMe-*myo*-; compound 13, 1-deoxy-1-fluoro-*myo*-; compound 14, 3-deoxy-3-fluoro-*myo*-; compound 15, 3-deoxy-3-azido-*myo*-; compound 16, *myo*-inositol-2-phosphate; compound 17, DL-1,2-anhydro-*myo*-inositol; compound 18, D6-*myo*-inositol; compound 19, *myo*-inositol-1-phosphate; compound 20, none (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/396/bj3960287add.htm for structures). White area represents percentage of [^{3}H]PI formed when compounds were used at 25 μM, and black area represents percentage of [^{3}H]PI formed when compounds were used at 1 mM. All determinations were performed in triplicate.

Secondly, a cell-free system using membranes isolated from *E. coli* expressing TbPIS was used for all subsequent biochemical characterization of the recombinant protein. Under the conditions used, the PIS activity was linear over the first 2 h (results not shown), with an optimal pH range of 7–8 (results not shown). The TbPIS catalytic activity was dependent on the presence of Mg^{2+} and CDP-DAG (results not shown). With the assay conditions used in the present study, the K_m (app) for *myo*-inositol was found to be 2 μM (Figure 2C). This is significantly lower than that reported for native PISs from *Chlamydomonas reinhardtii* (0.2 mM) [30], turkey erythrocyte membranes (0.3 mM) [31], human placenta (0.28 mM) [32] and yeast (0.1 mM) [1].

Like previously described PISs [2,6], the recombinant TbPIS was able to catalyse head group exchange between inositol of pre-existing PI (soya bean) with free [^{3}H]*myo*-inositol; this activity was detected in the absence of CMP but was greatly enhanced (~5-fold) in the presence of 4 μM CMP (results not shown).

The specificity of the TbPIS was investigated with respect to *myo*-inositol; various inositol isomers and analogues were added to the standard assay mixture and their ability to compete with the [^{3}H]*myo*-inositol to form [^{3}H]PI was determined (Figure 2D). When 25 μM (12.5 times K_m) *myo*-inositol was added to the standard reaction mixture, the incorporation of [^{3}H]*myo*-inositol into [^{3}H]PI decreased to approx. 15% of normal as expected, showing that the unlabelled *myo*-inositol is competing with [^{3}H]*myo*-inositol. However, none of the other eight isomers of inositol [*muco*-, *neo*-, *epi*-, D(+)-*chiro*-, L(−)-*chiro*, *cis*-, *allo*- and *scyllo*-] or the inositol analogues (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/396/bj3960287add.htm for structures) were able to affect the incorporation of [^{3}H]*myo*-inositol into [^{3}H]PI. At 1 mM, only *neo*-, *epi*-, L(−)-*chiro*-, *allo*-, *scyllo*-, 1-deoxy-1-fluoro-*scyllo*-, 2-OMe-*myo*-, 1-deoxy-1-fluoro-*myo*-, 3-deoxy-3-fluoro-*myo*- and D_6-*myo*-inositol caused a reduction in the incorporation of [^{3}H]*myo*-inositol into [^{3}H]PI, suggesting that at this very high concentration they were able to interact with the TbPIS. From these findings, we propose that the orientation of the 1-, 3- and 4-hydroxyls may be important and represents the minimum recognition motif as shown in Supplementary Figure S1 at http://www.BiochemJ.org/bj/396/bj3960287add.htm.

The synthesis of PI is essential in bloodstream form *T. brucei*

PIS appeared to be a single copy gene per haploid genome in *T. brucei* in the genome sequence database (Sanger Centre), which was confirmed by Southern blot analysis (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/396/bj3960287add.htm). A schematic representation of the strategy employed for construction of the conditional double knockout in bloodstream form *T. brucei* is shown in Figure 3(A).

Attempts to create a double null mutant of *TbPIS* were unsuccessful, suggesting that *TbPIS* is an essential gene (see Supplementary data). Therefore, to allow creation of a conditional double knockout cell line, a Myc-tagged ectopic copy of *TbPIS* was integrated into the Δ*PIS::PAC* cell line via the pLew100 vector [30]. Although integration of the ectopic copy was successful, attempts to delete the second *TbPIS* allele were again unsuccessful (see Supplementary data, Figures S3 and S4 at http://www.BiochemJ.org/bj/396/bj3960287add.htm). One possibility postulated for this was that the C-terminal Myc tag encoded by the pLew100 vector was interfering with the TbPIS localization and/or activity. Therefore the Myc tag was exchanged for an HA tag, which in separate experiments appeared not to hinder the transcription/translation of the TbPIS.

The HA-tagged ectopic copy was electroporated into the Δ*PIS::PAC* cell line using the modified pLew100-*HA* vector and clones were selected with puromycin and phleomycin. Several Δ*PIS::PAC PIS-HA*^{Ti} clones (where 'Ti' is tetracycline-inducible) were obtained and the integration of the ectopic copy was initially confirmed by PCR using primers specific to the pLew100 vector (results not shown) and later by Southern blotting (Figure 3B,

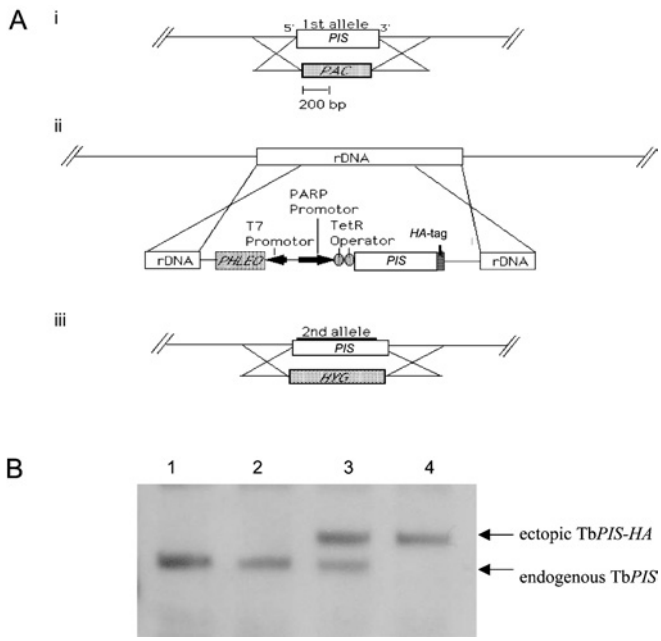

Figure 3 Construction of *PIS* conditional double knockout cell line

(**A**) Schematic representation of construction strategy used. (**i**) One allele of *PIS* was replaced by puromycin resistance gene (PAC) by homologous recombination, generating Δ*PIS::PAC* cell line, (**ii**) a tetracycline-inducible ectopic copy of *PIS* was introduced into the rDNA, generating *PIS-HA*^{Ti}Δ*PIS::PAC* cell line, (**iii**) while tetracycline induces the expression of the ectopic copy, the remaining allele was replaced by a hygromycin resistance gene by homologous recombination, resulting in conditional double knockout cell line *PIS-HA*^{Ti}Δ*PIS::PAC*/Δ*PIS::HYG*. (**B**) Confirmation of genotype of *T. brucei PIS* conditional double knockout cell line. Southern-blot analysis of HinfI-digested genomic DNA (~2 μg) from wild-type *T. brucei* cells (lane 1), Δ*PIS::PAC* (lane 2), *PIS-HA*^{Ti}Δ*PIS::PAC* (lane 3) and *PIS-HA*^{Ti}Δ*PIS::PAC*/Δ*PIS::HYG* (lane 4) cells; the *PIS* ORF probe shows allelic *TbPIS* at 1 kb and the ectopic copy *TbPIS-HA*^{Ti} at approx. 1.2 kb.

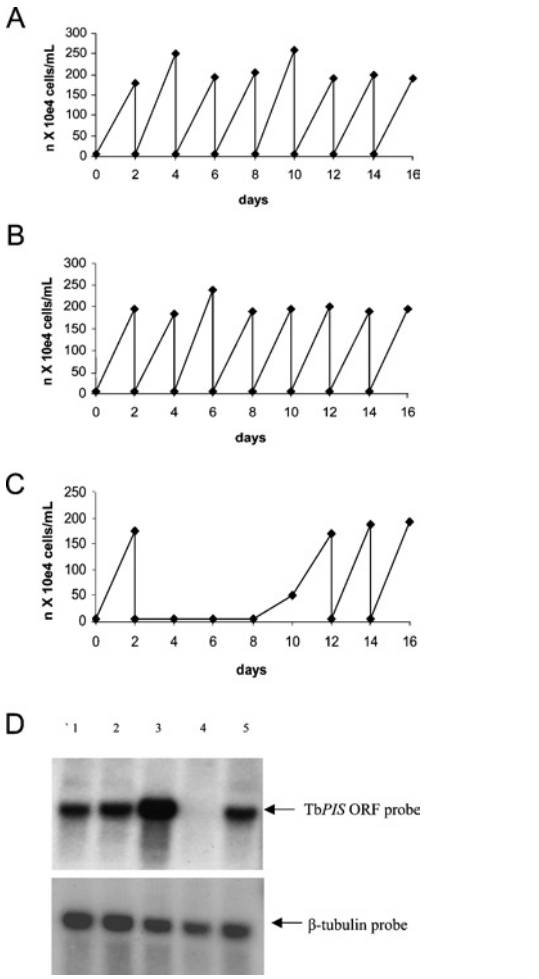

Figure 4 Growth curves and Northern blot of the *TbPIS* conditional double knockout cell line

Cells were washed with tetracycline-free HMI-9, and transferred to tetracycline-free medium and counted daily; experiments were performed on three independent occasions, and one set of data is shown that is representative of all experiments. Growth curves are shown for wild-type cells in this medium (**A**), *TbPIS* conditional double knockout with added tetracycline (**B**), and *TbPIS* conditional double knockout without tetracycline (**C**). (**D**) The Figure shows a Northern blot of total RNA from procyclic cells (lane 1), wild-type cells (lane 2), *TbPIS* conditional double knockout with tetracycline on day 2 (lane 3), *TbPIS* conditional double knockout without tetracycline on day 2 (lane 4) and *TbPIS* conditional double knockout without tetracycline on day 14 (lane 5). The blot was either probed with *TbPIS* ORF or β-tubulin, as detailed in the Experimental section.

lane 3). Transcription of this ectopic copy was induced by addition of tetracycline to the medium prior to deletion of the second allele of *TbPIS*. Deletion of both endogenous alleles was confirmed by Southern blotting, resulting in the cell line Δ*PIS::PAC*/*PIS::HYG*/*PIS-HA*^{Ti} (Figure 3B, lane 4).

The conditional double knockout Δ*PIS::PAC*/*PIS::HYG*/*PIS-HA*^{Ti} cell line was used to investigate the dependence of bloodstream form cells on the synthesis of PI. After being washed three times in tetracycline-free medium, cells were incubated in the same medium either in the absence or presence of tetracycline. The initial concentration of cells was 5×10^4 ml^{-1} and the cells were diluted only when the concentration was between 1 and 3×10^6 ml^{-1}, which was normally every second day. The conditional double knockout cells displayed normal growth rates in the presence of tetracycline when compared with wild-type cells (Figures 4A and 4B). In the absence of tetracycline, the cells grew normally for the first 2 days; however, after this time, cell numbers decreased and a distinct morphological change was observed (see Supplementary Figure S5 at http://www.BiochemJ.org/bj/396/bj3960287add.htm); concurrently increasing levels of cell debris were observed, suggesting cell death (Figure 4C). However, after 9–10 days in the absence of tetracycline, live cells were observed, suggesting that a small proportion of the dying cells were able to spontaneously resume normal growth rates. To determine whether this was due to an adaptation (e.g. uptake of sufficient PI from the extracellular environment) or due to re-expression of the *TbPIS* ectopic copy, transcription levels of the *TbPIS* was investigated. After culturing in the absence of tetracycline for 2 days, there was no detectable transcript of the *TbPIS* ectopic copy. However, in the cells that spontaneously resumed growth in the absence of tetracycline, there was a clear *TbPIS* transcript (Figure 4D, lane 5). This suggests that these revertent cells were able to overcome the tetracycline control, enabling the cells to resume expression of TbPIS. This spontaneous recovery of *T. brucei* conditional double knockout cell lines grown in the absence of tetracycline has been described for other essential genes in [13,21,22] and is due to loss of tetracycline control, which in one case was shown to be due to the deletion of the tetracycline repressor gene [22].

Biochemical phenotype of *TbPIS* conditional knockout cells

The biochemical phenotype of the *TbPIS* conditional knockout cells was investigated by quantitative and qualitative analyses of inositol-phospholipids by GLC-MS and MS respectively and

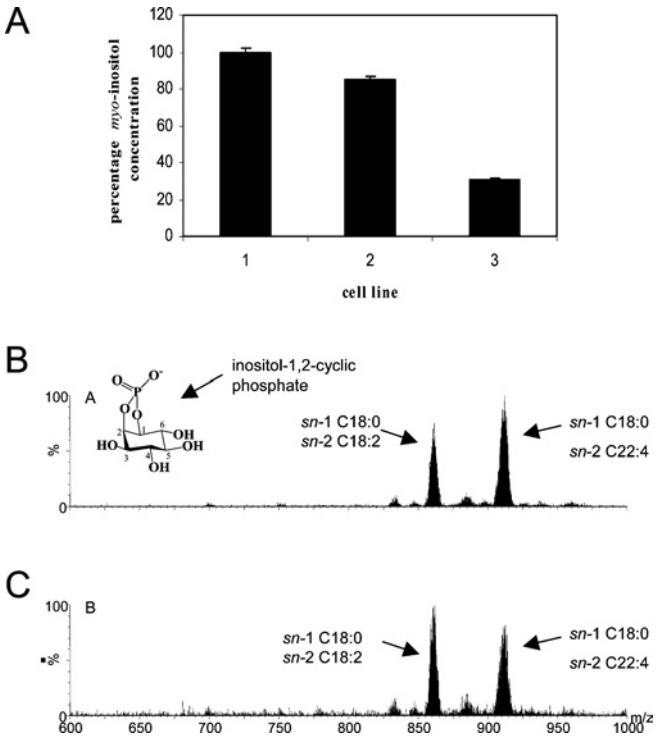

Figure 5 PI analyses of *TbPIS* conditional double knockout cells

(**A**) Lipid *myo*-inositol levels were quantified by GLC-MS as detailed in the text from wild-type cells (cell line 1), *TbPIS* conditional double knockout cells grown in the presence of tetracycline (cell line 2) and *TbPIS* conditional double knockout cells grown in the absence of tetracycline (cell line 3); 100 % represents 55 pmol of *myo*-inositol/5 $\times$ 10^4 cell equivalents. Inositol-phospholipids were qualitatively analysed by ESI-MS/MS using parent-ion scanning of *m/z* 241 (the collision-induced fragment of PI, inositol-1,2-cyclic phosphate) in negative ion mode from either (**B**) wild-type cells or (**C**) *TbPIS* conditional double knockout cells grown in the absence of tetracycline.

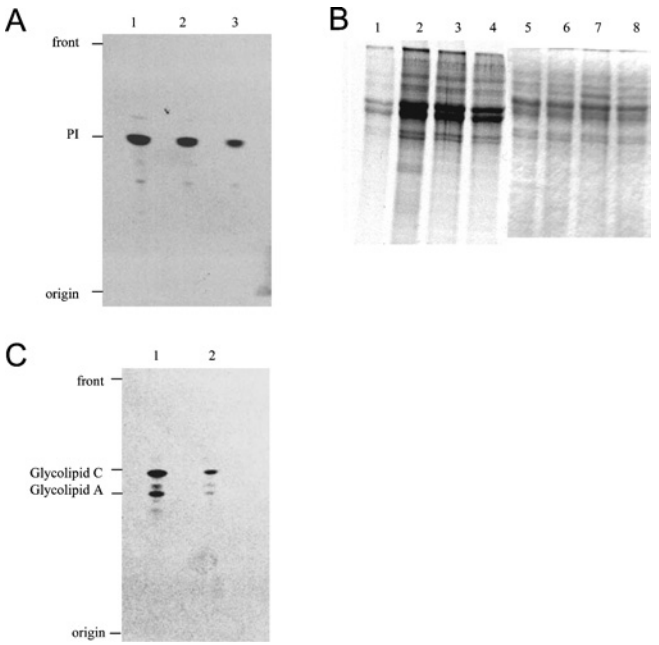

Figure 6 Biochemical phenotype analyses of the *TbPIS* conditional knockout cells

Prior to labelling, all cells were grown in tetracycline-free medium for 2 days; when tetracycline was necessary, it was added daily at 1 μg/ml. (**A**) Cells were labelled with [^{3}H]*myo*-inositol, lipids were extracted and detected as detailed in the Experimental section. Lane 1, wild-type cells; lane 2, *TbPIS* conditional double knockout cells grown in the presence of tetracycline; and lane 3, *TbPIS* conditional double knockout cells grown in the absence of tetracycline. (**B**) Cells were labelled with [^{35}S]methionine, and protein samples were prepared, separated and detected by using either autoradiography (lanes 1–4) or Coomassie Blue staining (lanes 5–8). Lanes 1 and 5, wild-type cells pre-incubated for 5 min with 60 μg/ml cycloheximide prior to labelling; lanes 2 and 6, wild-type cells; lanes 3 and 7, *TbPIS* conditional double knockout cells grown in the presence of tetracycline; and lanes 4 and 8, *TbPIS* conditional double knockout cells grown in the absence of tetracycline. (**C**) Cells were labelled with [^{3}H]mannose, and lipids were extracted and detected as detailed in the Experimental section. Lane 1, wild-type cells; and lane 2, *TbPIS* conditional double knockout cells grown in the absence of tetracycline.

in vivo labelling. For all of these analyses, the conditional double knockout cells were grown in the absence or presence of tetracycline for 2 days and the results were compared with those from wild-type cells grown under the same conditions.

Total phospholipids were extracted from wild-type cells and conditional double knockouts grown either in the presence or absence of tetracycline for 2 days and the amount of *myo*-inositol was determined by GLC-MS. As shown in Figure 5(A), there was a slight decrease in the amount of lipid containing *myo*-inositol in the conditional double knockouts grown in the presence of tetracycline when compared with the wild-type cells. This may be due to the ectopic copy of *PIS* either not being as enzymatically efficient because of the HA tag or not being regulated to the same level as the endogenous *PIS*. When the conditional double knockout cells were grown in the absence of tetracycline for 2 days, there was a significant decrease in the amount of lipid-bound *myo*-inositol to approx. 30 % of that present in wild-type cells (Figure 5A).

To ascertain if there was a global decrease of PI species within the conditional double knockout cells grown under non-permissive conditions, or if only specific species had decreased, inositol-phospholipids were qualitatively analysed by ESI-MS/MS using parent-ion scanning of *m/z* 241 in negative ion mode, the indicative collision-induced fragment of all PI species (inositol-1,2-cyclic phosphate). Two peaks were identified as $C_{18:0}/C_{18:2}$ and $C_{18:0}/C_{22:4}$; both have been previously identified as major forms of DAG PI from bloodstream form *T. brucei* [33,34]. There was no significant change in the ratios of peaks when conditional double knockout cells grown under non-permissive conditions for 2 days were compared with wild-type cells (compare Figures 5B and 5C). Also, there was no apparent change in the other phospholipids as determined by ESI-MS (results not shown). These results coupled with the GLC-MS analysis show that there was an overall decrease in the amount of inositol-containing phospholipids, which appeared to affect all species to a similar extent. This reduction in PI may have a detrimental effect on cell signalling and/or GPI anchor biosynthesis.

In vivo labelling with [^{3}H]*myo*-inositol suggested that the total amount of radioactivity incorporated into the lipid fraction of the conditional double knockout cells grown in the presence or absence of tetracycline was reduced to approx. 90 and 27 % respectively when compared with wild-type cells. Analysis of the [^{3}H]lipids by HPTLC revealed the same [^{3}H]lipid, which had an identical R_f to PI (Figure 6A, lanes 1–3). These results, together with those from the GLC-MS *myo*-inositol analysis, show that in the conditional double knockout cells grown in the absence of tetracycline, PI synthesis had substantially decreased, thus confirming that the deleted gene is a functional PIS in bloodstream form *T. brucei*. However, some residual PIS activity remained within these cells to account for the [^{3}H]PI, albeit at a reduced level. As there was no detectable *TbPIS* mRNA detectable after the removal of tetracycline for 2 days (Figure 4D, lane 4), it must take at least this time for total cellular PIS loss due to protein turnover

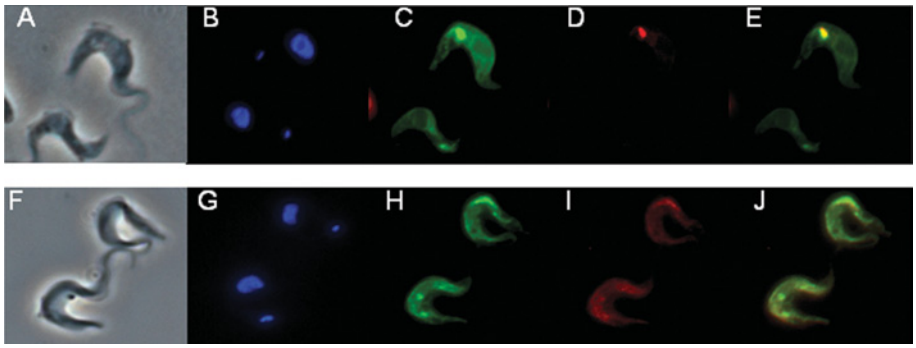

Figure 7 Subcellular localization of PIS–HA^{Ti} in bloodstream form *T. brucei* cells

Cells expressing PIS–HA^{Ti} were co-stained for the HA epitope and cellular markers. Co-staining with antibodies against the Golgi protein TbGRASP is shown in (**A**–**E**). (**A**) Phase contrast image; (**B**) DAPI staining; (**C**) HA epitope staining and FITC detection; (**D**) TbGRASP staining and TRITC detection; and (**E**) merged image of HA detection and TbGRASP detection. Co-staining with antibodies against the ER protein BiP is shown in (**F**–**J**). (**F**) Phase contrast image; (**G**) DAPI staining; (**H**) HA epitope staining and FITC detection; (**I**) BiP staining and TRITC detection; and (**J**) merged image of HA detection and BiP detection.

and dilution by cell division, thus also explaining why the cells are still alive at the point.

To confirm that the cells were still viable at the point of labelling, their ability to synthesize protein was investigated in parallel with the *myo*-inositol *in vivo* labelling. The wild-type cells were able to incorporate a significant amount of [^{35}S]methionine into newly synthesized protein (Figure 6B, lane 2) as compared with those pretreated with cycloheximide, a known protein synthesis inhibitor (Figure 6B, lane 1). The conditional knockout cells grown in the presence or absence of tetracycline for 2 days showed similar amounts of [^{35}S]methionine incorporation as compared with wild-type cells (Figure 6B, compare lanes 2–4), indicating that at the point of *in vivo* labelling, the cells were able to synthesize protein. Therefore *in vivo* labelling clearly shows that when the *PIS* conditional double knockout cells are grown under non-permissive conditions for 2 days, their ability to synthesize PI has decreased as a direct result of the deletion of *PIS* and not because the cells have lost viability.

To investigate any knock-on effects of the decrease in PI synthesis, the status of GPI biosynthesis was assessed. The conditional double knockout cells grown in the absence or presence of tetracycline for 2 days were labelled with [^{3}H]myristate and [^{3}H]mannose and compared with labelled wild-type cells. When labelled with [^{3}H]myristate, all three cell lines (wild-type, conditional double knockout with/without tetracycline) showed similar amounts of ^{3}H-labelled lipid species (results not shown), suggesting that the deletion of *PIS* has had no detrimental effects on general lipid biosynthesis at this time point. *In vivo* [^{3}H]mannose labelling of the wild-type cells showed the expected mature GPI glycolipids A and C (Figure 6C) which have been described previously [35,36]. When the conditional double knockout cells grown under non-permissive conditions were compared with the wild-type cells, there was a significant decrease in the amount of labelled glycolipids A and C (Figure 6C). This shows that the decrease in PI synthesis due to the deletion of *PIS* has had a direct knock-on effect on the GPI biosynthetic pathway. *In vivo* labelling with [^{3}H]*myo*-inositol showed that although PIS activity had decreased, there was some residual activity remaining (Figure 6A). This remaining PIS activity would continue to decrease until PI synthesis ceases or reaches a critical point where there is insufficient PI available for GPI synthesis. As a result, newly synthesized GPI-anchored VSG would also decrease, a situation that has previously been suggested to be lethal to the parasite [13–15]. This is similar to the chemical and genetic validation of the *T. brucei* GPI pathway, where soon after GPI biosynthesis had slowed by greater than 90 %, the parasites die [13–15].

TbPIS is localized in the ER and Golgi

To investigate the subcellular location of PIS in *T. brucei* bloodstream form cells, a tetracycline-inducible ectopic copy was introduced in the rRNA locus using the expression vector pLew82, which encodes a C-terminal HA tag. Integration of the ectopic copy was confirmed by PCR, Southern and Northern blotting (results not shown). Primarily this cell line (*TbPIS-HA*^{Ti}) was used for immunofluorescence and the results are presented here; however, when the conditional double knockout cell line Δ*PIS::PAC*/*PIS::HYG*/*PIS-HA*^{Ti} was created, immunofluorescent detection of the HA tag was repeated and found to be identical with that observed for the *TbPIS-HA*^{Ti} cell line. Transcription of the *TbPIS-HA*^{Ti} ectopic copy was induced by the addition of tetracycline to the medium. The TbPIS–HA^{Ti} protein was detected by immunofluorescence using a primary antibody against the HA tag and a secondary antibody that was FITC-conjugated. Two concomitant signals were observed (Figures 7C and 7H); one was a distinct signal positioned between the nucleus and kinetoplast, suggesting a Golgi location. The second was a reticular cytoplasmic signal with some perinuclear staining indicative of ER. When the cells were co-stained with rat antibodies against the HA epitope and rabbit antibodies against TbGRASP, a known Golgi protein [24], we observed almost complete overlap of the two signals, suggesting that TbPIS is found in the Golgi (Figure 7E). We also co-stained the cells with rat antibodies against the HA epitope and rabbit antibodies against the *T. brucei* ER-luminal protein BiP [23]. Both proteins displayed reticular staining throughout the cytoplasm, with some perinuclear staining. There was significant co-localization between TbPIS and BiP, suggesting that TbPIS is also found in the ER (Figure 7J). Localization of TbPIS to the ER was not totally unexpected; to date PISs from other organisms have predominately, and in some cases solely, been found in the ER (e.g. [4,31,32]). A secondary location for TbPIS in the Golgi is consistent with localization of the yeast PIS, which is found in both the ER and Golgi [1,9].

Conclusions

The identity of a putative *TbPIS* identified in the *T. brucei* genome was confirmed by preliminary characterization of the recombinant protein expressed in *E. coli*. The TbPIS is found in the ER and

Golgi in the bloodstream form of the parasite. Through the creation of a conditional double knockout, the *TbPIS* gene was shown to be an essential gene in bloodstream form *T. brucei*, thus genetically validating this particular enzyme as a potential drug target against African sleeping sickness and potentially other diseases caused by protozoa. Quantitative and qualitative analyses as well as *in vivo* metabolic labelling of the conditional double knockout cells confirmed that there was a significant decrease in all the major PI species in the cell. This decrease had a detrimental effect on GPI biosynthesis, which would ultimately be lethal to the parasite.

Genetic validation of PI synthesis as an anti-trypanosomal target demonstrates that the bloodstream form of the parasite cannot compensate for the loss of *de novo* synthesis by scavenging from its environment *in vitro*. These results also suggest that biosynthetic steps upstream of PI synthesis may also be potential drug targets, a hypothesis we are currently investigating.

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