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A novel phospholipase from *Trypanosoma brucei*

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Summary

Phospholipase A_1 activities have been detected in most cells where they have been sought and yet their characterization lags far behind that of the phospholipases A_2, C and D. The study presented here details the first cloning and characterization of a cytosolic PLA_1 that exhibits preference for phosphatidylcholine (GPCho) substrates. *Trypanosoma brucei* phospholipase A_1 ($TbPLA_1$) is unique from previously identified eukaryotic PLA_1 because it is evolutionarily related to bacterial secreted PLA_1. A *T. brucei* ancestor most likely acquired the *PLA*$_1$ from a horizontal gene transfer of a *PLA*$_1$ from *Sodalis glossinidius*, a bacterial endosymbiont of tsetse flies. Nano-electrospray ionization tandem mass spectrometry analysis of *TbPLA*$_1$ mutants established that the enzyme functions *in vivo* to synthesize lysoGPCho metabolites containing long-chain mostly polyunsaturated and highly unsaturated fatty acids. Analysis of purified mutated recombinant forms of $TbPLA_1$ revealed that this enzyme is a serine hydrolase whose catalytic mechanism involves a triad consisting of the amino acid residues Ser-131, His-234 and Asp-183. The *TbPLA*$_1$ homozygous null mutants generated here constitute the only *PLA*$_1$ double knockouts from any organism.

Introduction

Phospholipase A_1 (PLA_1) (EC 3.1.1.32) specifically hydrolyses the *sn*-1 acyl esters from phospholipids (PLs) producing free fatty acids (FAs) and lysophospholipids (lysoPLs). PLA_1 activities have been described in numerous cell types and tissues from a diverse range of organisms, but despite their apparent ubiquity and diversity relatively very few PLA_1s have been cloned and characterized.

A minority of PLA_1s cloned thus far possess a cytosolic subcellular localization, all three of which are homologues of one another and specifically utilize phosphatidic acid (GPA)[1] as substrate (Higgs *et al*., 1998; Tani *et al*., 1999; Nakajima *et al*., 2002). PLA_1 enzymes whose primary amino acid sequences exhibit homology with neutral lipase sequences can be categorized into a separate subgroup; these PLA_1, like their lipase homologues, are secreted from their cell (Soldatova *et al*., 1993; Hoffman, 1994; King *et al*., 1996; Sato *et al*., 1997; Watanabe *et al*., 1999; Sonoda *et al*., 2002). The preferred substrates observed for this group of enzymes so far are GPA (Sonoda *et al*., 2002) and phosphatidylserine (GPSer) (Sato *et al*., 1997). Secreted PLA_1 from bacteria that are not homologous to either lipases or mammalian PA-PLA_1 have also been cloned (Givskov *et al*., 1988; Schmiel *et al*., 1998). Finally, two unrelated sequences cloned from *Arabidopsis thaliana* code for PLA_1s localized

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[1]The lipid nomenclature system used closely follows the new comprehensive classification, nomenclature, and chemical representation system for lipids recently developed by an international consortium (Fahy *et al*., 2005).

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to organelles (Ishiguro *et al.*, 2001; Noiriel *et al.*, 2004); a third (Kato *et al.*, 2002) is homologous to mammalian PA-PLA_1 (Higgs *et al.*, 1998).

The biological significance of any of the various PLA_1 forms is not well understood and has not been explored in depth. Mounting evidence, however, suggests that some PLA_1 act to generate bioactive lipid molecules, a role normally thought to be reserved for the phospholipases A_2, C and D. PS-PLA_1 secreted from platelets selectively forms the lipid mediator lysoGPSer (Sato *et al.*, 1997) which can effectively stimulate histamine release from mast cells (Hosono *et al.*, 2001). Also, a PLA_1 from *A. thaliana* has been shown to catalyse the release of linoleic acid, the initial and regulatory step in jasmonic acid biosynthesis, a multifunctional growth regulator in plants (Ishiguro *et al.*, 2001). Furthermore, two GPA-utilizing PLA_1s are suspected to play a role in the regulation of intracellular membrane transport (Tani *et al.*, 1999; Nakajima *et al.*, 2002; Shimoi *et al.*, 2005). PLA_1 have also been theorized to co-ordinate with other phospholipases to form lipid messengers such as lysoGPA, which has been shown to be produced by a PLD/PA-PLA_1 concerted mechanism and to activate the cellular receptor EDG7/LPA_3 in Sf9 cells (Sonoda *et al.*, 2002).

Likewise, it has been postulated for decades that *sn*-2 FA cleavage from PLs may sometimes be mediated by concerted sequential PLA_1/LysoPLA activities (van den Bosch, 1980; Arthur, 1989; Burgoyne and Morgan, 1990; Badiani and Arthur, 1991; Pete *et al.*, 1996; Pete and Exton, 1996; Wang *et al.*, 1997; Ridgley and Ruben, 2001; Gauster *et al.*, 2005). A relevant consequence of this type of reaction could be, for example, the discharge of arachidonic acid (AA), which can act as a second messenger and precursor of eicosanoids (Gijon *et al.*, 2000).

The PLA_1/LysoPLA route towards the release of unsaturated FAs has been supported indirectly by *in vitro* studies using soluble subcellular fractions from bovine brain (Pete *et al.*, 1996) and from the kinetoplastid protozoan parasite *Trypanosoma brucei*, the infective agent responsible for African Sleeping Sickness (Ridgley and Ruben, 2001). Both of these studies observed the requirement of *sn*-1 acyl cleavage prior to the release of AA from *sn*-1-palmitoyl-*sn*-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine [GPCho(16:0/20:4)]. In support of this, a soluble PLA_1 and a soluble LysoPLA, the latter of which selectively deacylates lysoGPCho(−/20:4), have been chromatographically purified from bovine brain (Pete *et al.*, 1994; Pete and Exton, 1996). Moreover, LysoPLA activities in guinea pig microsomes have been observed to show preference for lysoGPCho(−/20:4) and lysoGPEth(−/20:4) (Arthur, 1989; Badiani and Arthur, 1991). Sequence identification of the enzymes responsible for the PLA_1 and LysoPLA activities from any of the examples outlined above has not been made.

The fate of unsaturated FAs liberated from lipids in *T. brucei* is not certain, but free AA has been implicated in regulating calcium mobilization in the cells (Eintracht *et al.*, 1998; Catisti *et al.*, 2000). Alternatively, the acid has been shown to serve as a precursor for prostaglandin biosynthesis in *T. brucei* (Kubata *et al.*, 2000). Though studies on the utilization of unsaturated FAs in *T. brucei* have been initiated, none of the phospholipases responsible for FA release from PLs have been identified. However, *sn*-1 FA cleavage from GPCho by *T. brucei* homogenates has been observed to be robust and optimal at pH 6.0–8.5; and this activity was attributed to PLA_1 activity in this organism (Tizard *et al.*, 1978a; Hambrey *et al.*, 1981; Opperdoes and van Roy, 1982). The homogenates possessed PLA_1 activity that was 10-fold greater than its LysoPLA activity, though both activities appeared to originate from the same soluble enzyme fraction (Sage *et al.*, 1981). A 380-fold enrichment in PLA_1 activity was achieved and the enzyme was purported to be 26 kDa (Hambrey *et al.*, 1984). Fractions enriched in PLA_1 activity were unable to cleave myristate from the

glycosylphosphatidylinositol anchor of the variant surface glycoprotein (Hambrey *et al.*, 1986).

To date, the only cloned and characterized phospholipase in *T. brucei* is GPI-PLC, which is a phospholipase C implicated in the cleavage of the variant surface glycoprotein from its glycosylphosphatidylinositol anchor (Hereld *et al.*, 1988; Carrington *et al.*, 1998; Gruszynski *et al.*, 2006). This report details the identification and characterization of a cytosolic PLA_1 from *T. brucei* that deacylates GPCho containing long polyunsaturated and highly unsaturated FAs.

Results

Identification and cloning of a *T. brucei PLA$_1$* gene that has a prokaryotic origin

In order to identify putative *PLA_1* genes in the *T. brucei* genome, a comprehensive search for phospholipases and lipases was carried out using the *T. brucei* genome database (GeneDB, Sanger Institute Pathogen Sequencing Unit, strain 927). Thirteen genes encoding putative lipolytic enzymes were identified, most of which contained the lipase consensus pattern [LIV] – {KG} – [LIVFY] – [LIVMST] – G – [HYWV] – S – {YAG} – G – [GSTAC] in their deduced amino acid sequence. The predicted protein sequence of each gene was subjected to local alignment analysis. Results revealed that one peptide sequence from *T. brucei* showed sequence similarity to PhlA, a secreted PLA_1 from the bacterium *Serratia liquefaciens* (Givskov *et al.*, 1988). The *T. brucei* phospholipase A_1 ($TbPLA_1$) was only homologous to bacterial PLA_1 isoforms, including YplA, a PhlA homologue from *Yersinia enterocolitica* (Schmiel *et al.*, 1998).

Very interestingly, the homologue with the greatest overall similarity to $TbPLA_1$ is a putative PLA_1 from the proteobacterium *Sodalis glossinidius* (morsitans strain) (Toh *et al.*, 2006), an intracellular endosymbiont of *Glossina* (tsetse) flies, the insect vector host of *T. brucei*. There are no eukaryotic homologues of $TbPLA_1$, including in two other kinetoplastid members *Trypanosoma cruzi* and *Leishmania* spp., the aetiologic agents of Chagas disease and Leishmaniasis respectively. However, orthologues of $TbPLA_1$ were found in existing GeneDB coding sequences from the livestock-infective *Trypanosoma congolense* and *Trypanosoma vivax*. $TbPLA_1$ does not share similarity to neutral lipases nor other previously reported eukaryotic PLA_1 sequences, apart from possessing a lipase consensus motif.

Using sequence-specific primers, putative *$TbPLA_1$* was amplified from *T. brucei* genomic DNA (Lister 427 strain) by PCR and the 903 bp open reading frame (ORF) sequenced (GenBank™ accession number AJ716154). An alignment of the $TbPLA_1$ predicted amino acid sequence with those of the other *Trypanosoma* spp. and bacterial homologues is shown (Fig. 1). The deduced primary sequence of $TbPLA_1$ shares 21.5%, 18% and 18% identity with its homologues from *S. glossinidius*, *S. liquefaciens* and *Y. enterocolitica* respectively; whereas *T. congolense* and *T. vivax* possess 41% and 37% identity with $TbPLA_1$ respectively.

The bacterial sequences possess a signal sequence used in targeted secretion that appears to be absent or very altered and unrecognizable (by the SIGNALP algorithm) in the trypanosome sequences. This finding was the first suggestion of an intracellular localization for $TbPLA_1$. All in all, these results provide the first evidence that $TbPLA_1$ is a unique eukaryotic PLA_1 because it is solely related to PLA_1 from bacteria. Furthermore, the high similarity of the $TbPLA_1$ sequence to that of a bacterial endosymbiont of the vector responsible for transmission of *T. brucei* parasites to mammals strongly suggests that a *T.*

brucei ancestor acquired the *PLA*$_1$ gene through horizontal gene transfer (HGT) during cohabitation with the bacterium inside an infected tsetse fly.

Expressed and purified recombinant TbPLA$_1$ exhibits *sn*-1 exclusivity

To determine whether TbPLA$_1$ could cleave FAs esterified to the *sn*-1 position of PLs, a source of pure and active protein with which to carry out *in vitro* analysis was sought using recombinant protein expression techniques. The entire *TbPLA*$_1$ ORF was subcloned into the expression vector *pET15b*. Subsequent transformation of *Escherichia coli* and induction of *TbPLA*$_1$ expression by IPTG produced *de novo* synthesized soluble TbPLA$_1$ with an N-terminal His·tag (Fig. 2A, lane 3). The soluble enzyme was purified to homogeneity using nickel affinity chromatography followed by anion exchange (Fig. 2A, lanes 6 and 7). The pure His·tag-cleaved protein consistently migrated as a larger molecular weight protein by SDS-PAGE than expected from its predicted 34.80 kDa size, though peptide mass fingerprinting and MALDI-TOF mass spectrometry analysis shows that the protein is indeed TbPLA$_1$ with a nominal molecular weight of 34.98 kDa respectively (data not shown).

The pH dependence for recombinant TbPLA$_1$ was assessed using the Bis-BODIPY® FL-C$_{11}$-PC assay. Results demonstrated that the enzyme shows highest specific activity between pH 6.0 and 8.5, and that enzyme activity is abolished at low pH (Fig. 2B). The optimal activity was detected at pH 7.0 and this pH was used in all other subsequent measurements of TbPLA$_1$ activity.

The regiospecificity of cleavage for recombinant TbPLA$_1$ was initially examined by incubating it with radiolabelled GPCho(16:0/[^{3}H]18:0). This substrate was presented to the enzyme in the form of TX-100/GPCho mixed micelles. Both the formation of free [^{3}H]18:0 acid and lysoGPCho(−/[^{3}H]18:0) was monitored as a function of TbPLA$_1$ concentration (Fig. 2C). Results clearly show that lysoGPCho(−/[^{3}H]18:0) formation mirrors the metabolism of GPCho(16:0/[^{3}H]18:0), indicating *sn*-1 activity. TbPLA$_1$ apparently has no LysoPLA$_2$ activity because the resultant product from the first reaction, lysoGPCho(−/[^{3}H]18:0), cannot serve as a substrate for any subsequent reactions, based on the observation that [^{3}H]18:0 acid from position *sn*-2 was not detected. When Ca^{2+}, sometimes required as a cofactor for soluble PLA activity, was included in the assay, no [^{3}H]18:0 acid was seen to accumulate and no enhancement of *sn*-1 activity was observed. Taken together, these results indicated that recombinant TbPLA$_1$ hydrolyses FA esters exclusively at the *sn*-1 position of the glycerol backbone of GPCho.

TbPLA$_1$ most efficiently hydrolyses GPCho molecules

The ability of recombinant enzyme to hydrolyse *sn*-1 hydrocarbon chains of neutral lipid substrates and PLs containing different headgroups was investigated by quantifying any free FA reaction products by gas chromatograph mass spectrometer (GC-MS) after their derivatization into methyl esters. A summary analysis of the reaction products from an assortment of lipid substrates indicated that purified TbPLA$_1$ deacylated a variety of choline-containing PLs with greater efficiency than PLs containing ethanolamine, inositol, serine, or just phosphorous at the *sn*-3 position (Table 1). When acyl chain length is held constant with palmitate, substrate preference was observed, in decreasing order, as GPCho(16:0/16:0)> GPSer(16:0/16:0)>>GPA(16:0/16:0)=GPEth(16:0/16:0), the latter two were hydrolysed at around 1/10 the rate of GPCho(16:0/16:0).

TbPLA$_1$ displayed some LysoPLA$_1$ activity towards lysoGPCho(14:0/−) that amounted to 14.5% and 16.0% of its PLA$_1$ activity towards GPCho(14:0/18:0) and GPCho(14:0/14:0) respectively. A variety of molecular species of GPCho were hydrolysed with average rates between 54.0 and 120.2 μmol min^{-1} mg^{-1}. Diacylglycerol (DG) and bovine liver GPIns

showed exceedingly poor substrate capabilities because their rate of hydrolysis was less than 1/100 the rate of GPCho even when 18.5-fold more enzyme was added. No fatty acyl hydrolysis from plasmalogen GPCho or from triacylglycerol (TG) could be detected. These results show that $TbPLA_1$ exhibits robust activity towards GPCho and that GPCho, and to a lesser extent GPSer, is the preferred substrate for the enzyme *in vitro*.

$TbPLA_1$ activity is mediated by a Ser-His-Asp catalytic triad

Although $TbPLA_1$ is not homologous to neutral lipases, its amino acid sequence contains a lipase consensus pattern that harbours a conserved GXSXG motif (Fig. 1), an indicator that $TbPLA_1$ may be a member of the serine hydrolase superfamily. The catalytic triad in these enzymes is usually occupied by a base residue (His), an acid (Asp) and a nucleophile (Ser), the latter of which is identifiable as the S in the GXSXG motif. The segments of sequence in which the base and acid residues lie are not as obvious due to the regions' generally lower degree of conservation, but the acid is often located well between the nucleophile and base. Based on this information it was predicted that Asp-183 could be an acid residue involved in $TbPLA_1$ catalysis (Fig. 1).

Nevertheless, two conserved aspartic acid residues in recombinant $TbPLA_1$ (Asp-63 and Asp-183), along with a third (Asp-232), which is conserved in the trypanosomes, were mutated to alanine by site-directed mutagenesis in order to compare activity to recombinant wild-type (WT) protein. Mutant enzyme activity was also examined after replacing the *T. brucei* GXSXG serine (Ser-131) and the only conserved histidine residue (His-234) to alanine. All of the mutant $TbPLA_1$ proteins were expressed and purified at the same levels as WT $TbPLA_1$ with the exception of the D183A mutant, which produced fourfold less protein (Fig. 3). The activity of the D63A and D232A mutants maintained an average of 89% and 67% of recombinant WT activity respectively, implying that the presence of these residues is not obligatory for $TbPLA_1$ activity. The $TbPLA_1$ activity measured for the D183A, H234A and S131A mutants was less than 5% of the activity observed for the WT enzyme, but nearly equal to that of the no-enzyme control (Fig. 3). The negation of activity for three enzymes containing individual serine, histidine and aspartic acid residue mutations suggests that hydrolysis of substrate was prevented due to an interruption of a charge relay system that is known to be invoked by the Ser-His-Asp catalytic triads in serine hydrolases.

Generation of *$TbPLA_1$* mutants

As no PLA_1 has ever before been directly studied *in vivo* in its native cell, the next line of experiments sought to do just that in order to gain insight into $TbPLA_1$ function in the trypanosome itself. To set conditions for which to accomplish this, experiments which led to the creation of a double knockout of *$TbPLA_1$* were performed. Promoterless deletion constructs (*5′-puro^R-3′*, *5′-bsd^R-3′*) targeted to the 5′ and 3′ flanking regions of the *$TbPLA_1$* ORF were generated and genes for antibiotic resistance were cloned between the flanking regions. These constructs were used to stably transform PCF stage 'WT' cell lines, which are in fact transgenic clones that constitutively express T7 RNA polymerase and the tetracycline repressor protein (Wirtz *et al.*, 1994). *$TbPLA_1$* is a single copy gene (data not shown) and therefore two successive rounds of gene replacement by UTR-targeted homologous recombination with *5′-puro^R-3′* and *5′-bsd^R-3′* were sufficient in this case to replace the two alleles of *$TbPLA_1$*. Evidence for successful generation of the null mutant cell line Δ*pla1* is shown (Fig. 4A).

Genetic transformation was also carried out that served to generate cell lines which overexpressed *$TbPLA_1$* using the *T. brucei* tetracycline-inducible overexpression vector *pLEW100* (Wirtz *et al.*, 1999). *pLEW100/PLA1-myc* was engineered by cloning a recombinant form of *$TbPLA_1$* into *pLEW100*. Upon tetracycline induction, trypanosomes

transformed with *pLEW100/PLA$_1$-myc* produce an ectopic copy of TbPLA$_1$ with a C-terminal *myc* tag (PLA1-myc). In PCF cells, both the WT and Δ*pla1* cell lines were stably transformed with *pLEW100/PLA1-myc* (Fig. 4A). The respective *ovexPLA1-myc* overexpression and Δ*pla1 rescPLA1-myc* rescue clones, under tetracycline induction, produced recombinant ectopic *TbPLA$_1$* mRNA transcripts that were, on average, > 23-fold over the level of *TbPLA$_1$* transcripts in both their WT and respective uninduced counterpart cells (Fig. 4B). In culture, the *TbPLA$_1$* null mutants and tetracycline-induced *TbPLA$_1$* overexpression cell lines have no apparent phenotype in morphology or in growth rate (data not shown).

The various overexpression clones were examined for their ability to translate the overexpressed *TbPLA$_1$-myc* mRNA. A Western blot probed with anti-TbPLA$_1$ antibodies revealed that overexpression of PLA1-myc was minimal when compared with the level of mRNA overexpression (Fig. 4C). Compared with uninduced *ovexPLA1-myc* cells, its induced counterpart cells produced, on average, only 2.2 times the amount of TbPLA$_1$ protein (native PLA1 + PLA1-myc). This observed level of regulation at the level of mRNA production to protein translation is not surprising considering the potential damage a membrane-lytic enzyme such as TbPLA$_1$ could invoke if expressed at uncontrollable levels in the cell.

TbPLA$_1$ synthesizes unsaturated lysoGPCho metabolites *in vivo*

To be able to capture metabolites created via PLA$_1$ action *in vivo*, a highly sensitive detection method which enabled identification and characterization of global changes in *T. brucei* PLs was needed. To this end, nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS-MS) was employed to analyse individual PL classes present in WT, Δ*pla1*, *ovexPLA1-myc* and Δ*pla1 rescPLA1-myc* cells. In positive ion mode, collision-induced dissociation of PLs containing a choline head group results in the production of a unique fragment ion of *m/z* 184 (Kerwin *et al.*, 1994). Precursor ion scanning for *m/z* 184 from total lipids extracted from WT cells selectively detected $[M+H]^+$ ions of GPCho and sphingomyelin (Fig. 5A). Ions in the precursor *m/z* 184 ion scan spectra were annotated based on their $[M+H\text{-}NMe_3{}^{(+)}]^+$ daughter ion masses compared with that of their theoretical values.

The major peak areas in the *m/z* 184 precursor ion spectrum represent GPCho with the following number of FA carbons and their degrees of saturation (represented as their sum): *m/z* 834, C40:6; *m/z* 836, C40:5; *m/z* 838, C40:4; *m/z* 786, C36:2; *m/z* 788, C36:1; *m/z* 790, C36:0; *m/z* 774, alkylacyl C36:1 and/or alkenylacyl C36:0; *m/z* 772, alkylacyl C36:2 and/or alkenylacyl C36:1; *m/z* 810, C38:4; *m/z* 812, C38:3; *m/z* 814, C38:2.

Detection of lysoGPCho metabolites formed from either PLA$_1$ or PLA$_2$ activity was facilitated from *m/z* 184 precursor ion scanning of total lipids in a mass range (*m/z* 400–700) which excluded the dominating diacyl and alkylacyl/alkenylacyl GPCho species. Peak analysis of short range spectra from PCF WT cell lipids resulted in the identification of a set of $[M+H]^+$ lysoGPCho metabolites with the following FA constituents: *m/z* 568, C22:6; *m/z* 570, C22:5; *m/z* 572, C22:4; *m/z* 520, C18:2; *m/z* 522, C18:1; *m/z* 524, C18:0; *m/z* 548, C20:2; *m/z* 546, C20:3; *m/z* 544, C20:4 (Fig. 5B, panel 1). The abundance of these long-chain mostly polyunsaturated and highly unsaturated lysoGPCho metabolites is markedly lower than nearly all of the diacyl GPCho species as shown by its relative proportion to the minor diacyl species GPCho(28:1) and GPCho(28:0).

To be able to quantify the amount of long-chain unsaturated lysoGPCho intermediates synthesized by TbPLA$_1$ activity, lysoGPCho series peak intensities were also compared with internal standard peak intensities of non-natural lysoGPCho(17:0/−) and lysoGPCho(24:0/−)

added to each sample prior to lipid extraction (Fig. 5B, panel 1 inset). The calculated amounts of lysoGPCho metabolites from WT cellular lipids are tabulated as shown (Table 2). A permeating feature of the lysoGPCho molecules synthesized in *T. brucei* is that their acyl moiety is composed of a long and highly unsaturated chain of 22 carbons in nearly 50% of the metabolite population. This observation is consistent with the notion of the suspected presence of these FAs in some of the most abundant diacyl GPCho species (*m/z* 834–836), if the *sn*-1 position was occupied by C18:0 (Fig. 5A) (Patnaik *et al.*, 1993).

Equivalent systematic ESI-MS-MS analysis of PCF Δ*pla1* cells revealed that the levels of lysoGPCho metabolites in the null mutants decreased on average by 81% when compared with WT levels (Table 2) (Fig. 5B, panel 2 and inset). There was a 90% decrease in the number of moles of lysoGPCho(−/22:y), whereas lysoGPCho(−/18:y) and lysoGPCho(−/20:y) levels decreased by 72% and 74% in the *TbPLA*$_1$ null mutants respectively. The variety and abundance of GPCho(28:1)/GPCho(28:0) (Fig. 5B, panel 2) and the other diacyl GPCho molecular species (data not shown) in the null mutant remained unaltered. Overall, these results suggest that *in vivo* lysoGPCho biosynthesis in *T. brucei* is largely dependent upon TbPLA$_1$ activity. Furthermore, due to the *sn*-1 exclusivity of TbPLA$_1$ (Fig. 2 and Table 1), the FA of each lysoGPCho is esterified at the *sn*-2 position of these metabolites. It is unknown whether the small amount of lysoGPCho species still present in the null mutants is due to enzymatic synthesis *in vivo* by an alternative PLA, or whether they represent background levels of fragment ions randomly and mechanically formed from diacyl GPCho during the sample preparation and/or the ionization process. However, the observation that lysates of Δ*pla1* cells could not produce any lysoGPCho supports the idea that these cells are most likely totally devoid of unsaturated lysoGPCho molecules (Fig. 6).

Confirmation that TbPLA$_1$ mediates lysoGPCho metabolite synthesis *in vivo* comes from GPCho analysis in *ovexPLA1-myc* cells. Tetracycline-induced overexpression of *TbPLA*$_1$ in these cells resulted in an elevated abundance of lysoGPCho compared with either WT or non-induced *ovexPLA1-myc* cells (Fig. 5B, panels 3 and 4 and their respective insets). The levels of lysoGPCho intermediates from the (−/18:y) and (−/22:y) series in the overexpression cell line reached 3.0 and 3.2 times higher than normal PCF levels of these metabolites respectively (Table 2). Interestingly, the (−/20:y) series intermediates only showed a modest (1.4-fold) increase after tetracycline induction.

TbPLA$_1$-mediated synthesis of *T. brucei* long-chain polyunsaturated and highly unsaturated lysoGPCho was also established using the PCF Δ*pla1 rescPLA1-myc* rescue clones. The Δ*pla1* ablated lysoGPCho(−/18:y) metabolite levels were nearly restored (98%) to WT levels upon induction with tetracycline in Δ*pla1 rescPLA1-myc* cells, whereas the non-induced cells continued to display the Δ*pla1* phenotype (Fig. 5B, panels 5 and 6 and their respective insets) (Table 2). The number of moles of lysoGPCho(−/20:y) and lysoGPCho(−/22:y) were brought back to 71% and 75% of normal levels in the PCF rescue cell line.

Data from ESI-MS-MS analysis of *TbPLA*$_1$ mutants collectively revealed a range of lysoGPCho metabolites whose production is at least partly regulated by the levels of TbPLA$_1$ in the cell. A comprehensive short range search for lysoGPEth, lysoGPSer, lysoGPIns and lysoGPA yielded negative results; this supports previous findings that lysoGPCho in *T. brucei* is the predominant lysoPL synthesized by this parasite (Dixon and Williamson, 1970; Patnaik *et al.*, 1993). The robust activity of TbPLA$_1$ towards GPCho both *in vitro* and *in vivo* strongly suggests that GPCho is the preeminent substrate for this enzyme.

The global PL composition of the *TbPLA*$_1$ mutants generated in this study was also examined, but the high mass range spectrums of diacyl GPCho, GPEth, GPSer and GPIns

from the *TbPLA$_1$* mutants remained unchanged compared with the composition of these lipids in WT cells (data not shown).

TbPLA$_1$ is constitutively expressed in *T. brucei* and its gene product is located in the cytosol

The first empirical indication that TbPLA$_1$ may be a cytosolic protein came from observing that a high-speed soluble fraction (obtained from centrifuging lysate at 100 000 *g* for 1 h) from WT cells, but not lysate from *TbPLA$_1$* null mutant cells, could quickly form lysoGPCho in our radiolabelled assay (data not shown). The observations that optimal TbPLA$_1$ activity occurs at a neutral pH and that sequence analysis suggests that this protein has lost its signal peptide also support the notion that TbPLA$_1$ resides in the cytosol. Therefore, to confirm the intracellular location of the native enzyme, the presence of TbPLA$_1$ in subcellular fractions of *T. brucei* was investigated. A Western blot of subcellular fractions probed with antibodies raised against the purified recombinant enzyme reveals that native TbPLA$_1$ is recognizable as a soluble protein that localizes entirely in the cytosolic protein fraction in both bloodstream (BSF) and PCF WT *T. brucei* (Fig. 7A, lanes 3 and 4). No TbPLA$_1$ is detected in the cytosolic fraction of PCF Δ*pla1* cells (Fig. 7A, lane 5), nor in the large granular or microsomal fractions of BSF WT cells (Fig. 7A, lanes 1 and 2).

In a further gesture to pinpoint TbPLA$_1$ subcellular location, Δ*pla1* cells transformed with an altered *pLEW100/PLA1-myc* engineered to contain *EGFP* fused at either the N-terminus (Δ*pla1 EGFP-PLA1-myc*) or C-terminus (Δ*pla1 PLA1-EGFP*) of *TbPLA$_1$* were generated. TbPLA$_1$ expressed as a fusion protein with EGFP upon tetracycline induction of Δ*pla1 PLA1-EGFP* and Δ*pla1 EGFP-PLA1-myc* cells. Evidence that full-length fusion proteins were produced by these cell lines was obtained by Western blot analysis. Anti-TbPLA$_1$ antibodies detected the fusion proteins in the expected 60 kDa range, whereas the WT TbPLA$_1$ was detected in its native 35 kDa range (Fig. 7B, upper panel). A copy of *pLEW100* that contained only *EGFP* was also introduced into the null mutants as a control cell line (Δ*pla1 EGFP*). Western blot analysis using anti-EGFP antibodies showed that Δ*pla1 EGFP* cells expressed non-fused EGFP (27 kDa) after tetracycline induction (Fig. 7B, lower panel). Both Δ*pla1 EGFP-PLA1-myc* and Δ*pla1 PLA1-EGFP* induced cells expressing the PLA1/EGFP fusion proteins fluoresced upon UV exposure in a pattern very similar to cells expressing EGFP only, which is known to be a soluble protein localized to the cytosol of cells in which it is expressed (Fig. 7C). The localization studies taken together thus suggest that TbPLA$_1$ functions by hydrolysing *sn*-1 FAs of GPCho molecules exposed to the neutral environment of the cytoplasm.

Discussion

This report describes the identification and characterization of a PLA$_1$ from *T. brucei*, the first phospholipase of type A to be identified from this parasite, and the only PLA$_1$ cloned from any protist. TbPLA$_1$ should be considered a true PLA$_1$ because it possesses high and specific activity towards *sn*-1 esters of diacyl PLs, exhibits LysoPLA$_1$ activity that is a fraction of its PLA$_1$ activity, and shows very little activity towards neutral lipid substrates.

TbPLA$_1$ is distinctive from previously identified PLA$_1$ from other eukaryotes because it is only homologous to prokaryotic extracellular PLA$_1$ enzymes. Outside of its own *Genus* the TbPLA$_1$ peptide sequence most resembles a PLA$_1$ homologue from *S. glossinidius*, a recently established bacterial secondary endosymbiont of the *Glossina* (tsetse) fly (Aksoy *et al.*, 1995). This finding together with an analysis of trypanosomatid phylogeny allows us to propose that a *T. brucei* ancestor acquired the *S. glossinidius PLA$_1$* gene through HGT after/during its adaptation to a parasitic lifestyle in its tsetse host. The *PLA$_1$* HGT is predicted to have occurred before the species in the '*T. brucei*' clade of lineages diverged from one

another, but after they diverged from the species in the clade containing *T. cruzi*, *T. lewisi* and *T. theileri* (Fig. 8). This hypothesis is supported by several observations and deductions. First, $TbPLA_1$ homologues are absent in *T. cruzi* or the *Leishmania* spp. These two kinetoplastids do not use *Glossina* as a host and it is unlikely that they acquired *PLA1* through vertical gene transfer from an early kinetoplastid ancestor and then subsequently lost it. Second, an *S. glossinidius PLA1* homologue is present in *T. congolense* and *T. vivax*, whose lineages diverge within the same clade as *T. brucei*. Third, *T. brucei*, *T. congolense* and *T. vivax* all infect *Glossina* flies. Last, both *T. lewisi* and *T. theileri*, which have been reported to possess very little PLA_1 activity (Tizard *et al.*, 1977a; 1980), are rooted to a clade outside of *T. brucei* and *T. congolense*, trypanosomes that have high PLA_1 activity (this study) (Tizard *et al.*, 1978b,c; Hambrey *et al.*, 1980). Unlike prokaryote–prokaryote HGT, HGT between prokaryote and eukaryote is still regarded as more rare and atypical, and it is most common in protists (Boucher and Doolittle, 2000; Field *et al.*, 2000; de Koning *et al.*, 2000). On the whole, the probable HGT that created trypanosome *PLA1* from a proteobacteria contributed to the evolution of the *T. brucei* lineage, and it supports the idea of the coevolution of trypanosomes with their hosts.

This study successfully employed both *in vitro* and *in vivo* analytical techniques to establish a correlation that GPCho is the preferred substrate class upon which $TbPLA_1$ acts. The results from this study also provide evidence that $TbPLA_1$ is an intracellular enzyme localized to the cytosol, which is in stark contrast to the fate of its secreted bacterial homologues. Therefore, in addition to its distinct evolution, $TbPLA_1$ is also a novel PLA_1 because it is the only cytosolic PLA_1 thus far discovered whose preferred substrates are GPCho molecules. In fact, of all the PLA_1 cloned and characterized across taxa, only the two organelle-associated PLA_1s from *A. thaliana* have been shown to be able to hydrolyse GPCho to some extent (Ishiguro *et al.*, 2001; Noiriel *et al.*, 2004). Just three other cytosolic PLA_1 enzymes have been cloned previously from other organisms, all of which are mammalian in origin and preferentially hydrolyse GPA (Higgs *et al.*, 1998; Tani *et al.*, 1999; Nakajima *et al.*, 2002). $TbPLA_1$, on the other hand, shows very little activity towards GPA.

GPCho is the major PL in *T. brucei*, constituting approximately 57% of all the PL species present in *T. brucei* PCF parasites. The entire GPCho pool is not, however, a potential source of substrate for $TbPLA_1$; roughly a quarter of the GPCho pool consists of non-hydrolysable plasmanyl/plasmenyl species (Patnaik *et al.*, 1993). Furthermore, in most eukaryotic cells GPCho is thought to be the major PL of the outer leaflet of the plasma membrane, a location which would render many GPCho substrates inaccessible to $TbPLA_1$. Though $TbPLA_1$ presumably has access to PLs on the inner leaflet of the plasma membrane, the presence of GPSer and GPEth, which are widely accepted to reside in large part on the inner leaflet of the plasma membrane in their plasmanyl/plasmenyl form, could also naturally limit to a certain extent the availability of GPCho to $TbPLA_1$ in this location. Though the amount of GPCho distributed along cytosolic surfaces in the cell is unknown, it is quite possible that the majority of $TbPLA_1$ substrates are part of the outer leaflets of organelles and vesicles, and this includes the ER, where GPCho is *de novo* synthesized.

An important component in this study was the desire to answer some fundamental questions concerning the nature of *T. brucei* PLA_1 activity. The absence of a crystal structure for any true PLA_1[2] enzyme precludes making deductions on their mechanisms of action without empirical evidence. As such, to better comprehend the structural basis for the functional

[2]Structures exist for two enzymes that can cleave *sn*-1 esters to some extent. Outer membrane phospholipase A, however, can cleave at both positions *sn*-1 and *sn*-2 of diacyl- or lysoPLs and thus is considered a phospholipase B (Nishijima *et al.*, 1977). The guinea pig pancreatic lipase-related protein 2 displays both triglyceride lipase and phospholipase A_1 activities, and shares 63% sequence identity to human pancreatic lipase (Withers-Martinez *et al.*, 1996).

characteristics observed for $TbPLA_1$, the identification of the catalytic residues involved in *sn*-1 ester hydrolysis were sought. Sitedirected mutagenesis of recombinant $TbPLA_1$ provided evidence that the catalytic mechanism employed by $TbPLA_1$ is similar to that of the serine hydrolases, in that its activity appears to rely on the concerted action of a catalytic triad composed of Ser-131, Asp-183 and His-234. In theory, loss of activity from any of the mutants could have been due to conformational changes or protein misfolding engendered by the substituted alanine during protein expression. However, due to the good understanding of the catalytic mechanisms used by hydrolases, and the residues involved, it is unlikely that the complete loss of activity from these soluble and well-expressed mutant proteins was caused by unintentional structural anomalies. Also, the orientation of the catalytic triad residues of $TbPLA_1$ is consistent with that of the other serine hydrolases where the residues appear in the order Ser-Asp-His. Accordingly, $TbPLA_1$ is the newest member of the serine hydrolase family.

The central discovery from this report is that $TbPLA_1$ mediates the synthesis of long-chain polyunsaturated and highly unsaturated lysoGPCho metabolites in *T. brucei*. Detecting $TbPLA_1$-dependent fluctuations of these metabolites was reliant upon creating *TbPLA*$_1$ double knockout and overexpression transgenic cells. This is the only PLA_1 double knockout from any organism, a poignant fact which explains why not much is understood about this class of enzymes. As such, there is no precedent study which directly detects and manipulates *in vivo* PLA_1 activity in its native cell as is described here. Biochemical analysis of the *TbPLA*$_1$ null mutants and various transgenic *TbPLA*$_1$ overexpression cell lines showed that depleted or increased levels of $TbPLA_1$ provoked lysoG-PCho metabolite ablation and elevation respectively, whereas alterations in other PL pools were not detected. Interestingly, the levels of the lysoGPCho(−/20:y) intermediates did not rise as much as the lysoGPCho(−/18:y) and lysoGPCho(−/22:y) intermediates during *TbPLA*$_1$ overexpression. Importantly, however, it should be acknowledged that although certain lysoGPCho species are more abundant than others in the mass spectra, this does not necessarily mean that they are synthesized at greater rates by $TbPLA_1$ because both LysoPLA and acyltransferases could show preference to further metabolize a particular series of lysoPL intermediate (Arthur, 1989; Pete and Exton, 1996). A consequence of this type of scenario would be that very little if any global increase in metabolites would be detected despite their increased synthesis.

Unsurprisingly, *TbPLA*$_1$ null mutants and overexpression cell lines maintained normal cell growth and revealed no apparent phenotype in culture. In *Leishmania major*, another trypanosomatid, the inability to *de novo* synthesize sphingolipids and ether PLs, principle components of membranes, were also viable and did not result in growth defects (Zufferey *et al.*, 2003; Denny *et al.*, 2004). Furthermore, the abundance of lysoGPCho in eukaryotic cells is very well regulated and normally very low (0.5–1.0% of total lipids), and their loss does not seem to affect viability in other cells (van den Bosch, 1980). The high specific activity fostered by $TbPLA_1$, coupled with its cytoplasmic localization, poses a threat to the cell if high levels of the enzyme were allowed to freely circulate and activate because it could potentially lyse the cell from within by producing excess FAs and lysoGPCho. Besides some protection provided by the plasmanyl/plasmenyl lipids present in *T. brucei*, tight regulation of lysoGPCho levels should be crucial for the normal functioning of *T. brucei*. It is shown here that tetracycline induction of both the overexpression and rescue cell lines resulted in very abundant overexpression of *TbPLA*$_1$ mRNA, but not substantial PLA_1 overexpression. This result suggests that lysoGPCho levels in *T. brucei* are at least in part regulated at the level of *TbPLA*$_1$ mRNA translation and protein synthesis. Regulation of lysoGPCho levels is also most likely mediated downstream of their formation by regulating the enzymes that can further metabolize lysoPLs. As part of a Lands cycle, a *T. brucei* acyltransferase could remodel lysoGPCho intermediates to form a new diacyl GPCho, or

alternatively a LysoPLA could remove the remaining FA. A search of the *T. brucei* database does highlight several putative acyltransferase and LysoPLA sequences, whose activities may account for the *T. brucei* LysoPLA and acyltransferase activities observed by us and others (Tizard *et al.*, 1977b; Samad *et al.*, 1988; Bowes *et al.*, 1993; Werbovetz and Englund, 1996).

One of the lysoGPCho metabolites detected in this study, lysoGPCho(−/20:4), has previously been shown to be an intermediate in a proposed sequential PLA_1/LysoPLA mechanism for the liberation of AA in *T. brucei*, an organism which is thought to lack PLA_2 activity (Ridgley and Ruben, 2001). Cytosolic PLA_2 enzymes, which have a marked specificity for *sn*-2 AA liberation from certain PLs, are quite identifiable at the sequence level by their C2 and α/β hydrolase domains (Six and Dennis, 2000), yet a search of the *T. brucei* genome database does not reveal any cytosolic PLA_2 homologues. It is worth mentioning that a soluble enzyme activity from *T. brucei* that may release *sn*-2 FAs has been purported to be due to a PLA_2 (Shuaibu *et al.*, 2001), but it is curious that the properties of this activity are very similar to the properties presented here for recombinant $TbPLA_1$. Other authors have initially reported PLA_2 activity in *T. brucei* before later proposing that the activity was due to a concerted PLA_1/LysoPLA mechanism (Eintracht *et al.*, 1998; Ridgley and Ruben, 2001). Thus, with long-chain PLA_2 activity possibly absent in *T. brucei*, the parasite potentially could have evolved a PLA_1/LysoPLA *sn*-2 deacylation system which could utilize its adapted $TbPLA_1$.

If $TbPLA_1$ is part of an alternative mechanism to release *sn*-2 FAs, its loss potentially could have been compensated for by other lipolytic activities. However, this study showed that redundancy at the level of lysoGPCho synthesis is not supported by *T. brucei*; both the lysate PLA_1 activity and ESI-MS-MS experiments indicated that lysoGPCho synthesis is severely and most likely totally compromised in *TbPLA*$_1$ null mutants. Redundancy in lysoGPCho formation could, however, exist at undetectable levels, levels that could be sufficient to supply the necessary levels of AA to the cell, if this is a critical activity. It is possible that a lysosomal PLA_1 activity is present in *T. brucei* (Opperdoes and van Roy, 1982), but this would most likely serve to degrade endocytosed PLs and not serve to recover lost cytosolic PLA_1 activity.

Though the results reported here bring us a step closer, it is still not proven that $TbPLA_1$ functions in a pathway to release AA from GPCho, nor if this AA release is essential for the cell. However, if redundancy is a reason why *TbPLA*$_1$ null mutants are still viable and show no growth phenotype, it most likely does not exist at the level of endogenous lysoGPCho(−/20:4) synthesis, but at the level of AA production and/or liberation from other lipids. In higher eukaryotes, both endogenous and exogenous linoleic acid ($C18:2\Delta^{9,12}$) can be desaturated, elongated and desaturated again to produce AA ($C20:4\Delta^{5,8,11,14}$). Both elongation and desaturation of FAs in trypanosomes has been observed (Dixon *et al.*, 1971). But, it has recently been shown that *T. brucei* uses three of its elongases (elongases 1–3) in BSF and PCF parasites to elongate pre-existing FAs to myristate (C14:0) and stearate (C18:0) respectively (Lee *et al.*, 2006); therefore, elongase 4, shown to elongate polyunsaturated FAs, may be the only elongase which may play a role in AA synthesis. Of course, AA can also be taken up directly by the cell from LDL-linked or albumin-linked lysoGPCho and non-esterified FAs in the blood, whose AA derivatives amount to 6.98% and 0.97% of the AA composition in human plasma respectively (Croset *et al.*, 2000). *T. brucei* LysoPLA activity has been shown to efficiently degrade exogenous lysoGPCho (Samad *et al.*, 1988; Werbovetz and Englund, 1996). Last, AA could conceivably be freed from neutral lipids by lipases.

It is evident that PL degradation by intracellular phospholipases and lysophospholipases is an exceedingly complex matter. This study has contributed significantly to the understanding of how $TbPLA_1$ functions at a biochemical and molecular level in *T. brucei*. Equally important is the fact that $TbPLA_1$ has emerged as a unique example of a PLA_1 enzyme.

Experimental procedures

Cloning of *TbPLA*$_1$

BLAST analysis identified a putative PLA_1 in the *T. brucei* genome. NdeI-containing forward (5′-atagtacggcatATGTCGGAATCCGTTGATCAGT-3′) and BamHI-containing reverse (5′-gacattaggatcctcTGAAGAACCGAACCCTCCG-3′) primers were used to PCR amplify *TbPLA*$_1$ from *T. brucei* genomic DNA using *Pfu* polymerase (small letters in primer sequences denote here and elsewhere an engineered restriction enzyme site and/or linker sequence). The PCR product was gel purified (Qiagen), ligated into a blunt-ended TOPO cloning vector (Invitrogen) and sequenced on both strands. Some purified PCR product was used as a probe in Southern blotting after fluorescein-labelling by random priming (Amersham Biosciences).

Expression and purification of *TbPLA*$_1$

TbPLA$_1$ excised from TOPO using NdeI and BamHI was subcloned into the pET15b expression vector (Novagen), resequenced and transformed into *E. coli* BL21 Gold cells (Novagen). The cells were grown at 37°C to an OD_{600} of 0.8 in 3.0 l of Luria–Bertani broth containing 50 μg ml^{-1} ampicillin, transferred to a 15°C shaking water bath, and induced overnight with 1.0 mM IPTG. Cells were harvested by centrifugation and resuspended in 40 ml of binding buffer (50 mM NaH_2PO_4 pH 8.4, 150 mM NaCl, 50 mM imidazole, 5% glycerol), lysed by a One-shot Cell Disrupter (Constant Systems), and the lysate was clarified by centrifugation at 10 000 *g*, 4°C for 25 min. The resultant supernatant was filtered (0.45 μm) and loaded onto a charged Hi-Trap nickel column (Amersham Biosciences) equilibrated with binding buffer. The column was washed extensively with binding buffer and recombinant protein was eluted in one step with 1.0 ml elution buffer (50 mM NaH_2PO_4 pH 8.4, 150 mM NaCl, 500 mM imidazole, 5% glycerol). Half of the eluate was immediately diluted 30-fold with anion exchange binding buffer (20 mM Tris-HCl pH 8.0, 5% glycerol) and loaded onto a Resource™ Q anion exchange column (Amersham Biosciences) equilibrated with binding buffer. The remaining half was digested with thrombin for 4 h at room temperature to cleave the His·tag from $TbPLA_1$ and then diluted and loaded onto the column. Gradient elution was achieved by using an AKTA Purifier pump (Amersham Biosciences) at 0.5 ml min^{-1} using anion exchange elution buffer (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 5% glycerol), and aliquots from the major peak were snap frozen. This 1 day procedure yields greater than 15.0 mg l^{-1} of highly active purified $TbPLA_1$. If needed, buffer exchange by dialysis was performed before certain experiments. Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin as a standard. His·tagcleaved $TbPLA_1$ was used in all experiments except where noted.

Radiolabelled PLA_1 assay

PLA_1 activity was determined by measuring the formation of lysoGPCho(−/[^{3}H]18:0) from GPCho(16:0/[^{3}H]18:0) (American Radiolabelled Chemicals, St Louis, MO) that was presented to the enzyme as mixed micelles. Mixed micelles were prepared as an 8× stock solution containing 1.2 mM GPCho(16:0/18:0) (specific activity 26.6 μCi μmol^{-1}) and 65.9 mM TX-100 (0.018 mole fraction) in 50 mM Tris-HCl, pH 7.0. The final assay mixture contained 50 mM Tris-HCl, pH 7.0, 75 mM NaCl, 150 μ_M GPCho substrate, and 0.63–60.0

ng of $TbPLA_1$ recombinant protein in a total volume of 40 μl. Enzyme was omitted from control samples. Reactions were initiated by the addition of substrate and incubated at 22°C for 5 min unless stated otherwise. $TbPLA_1$ reaction rates were linear up to 38 min. Lipids were extracted by the method of Bligh and Dyer (1959) and the lower phase was dried under nitrogen and redissolved in chloroform. Samples were applied to HPTLC Silica 60 plates (Merck) and developed in chloroform/methanol/water (10:10:3, v/v). The HPTLC plates were exposed to a BAS-TR 2040 imaging plate and the radiolabelled lipids detected with a FLA-5100 scanner (all from Fujifilm) and quantified using Advanced Image Data Analyzer (AIDA) densitometry software (Raytest).

Substrate specificity by GC-MS

An 80 μl reaction mixture containing 50 mM Tris-HCl pH 7.0, 100 mM NaCl and 3.75 mM substrate at a mole fraction of 0.036 with TX-100 was incubated with 100 ng enzyme at 22°C for 10 min. Reaction rates were linear with respect to time up to 35 min. Enzyme was omitted from control samples. Heptadecanoic acid (C17:0) internal standard (50 nmol) was added to each reaction tube before extraction of free FAs by the method of Dole (Dole and Meinertz, 1960). Fatty acid methyl esters (FAMEs) were prepared using diazomethane in diethyl ether and let to evaporate overnight before redissolving in 30 μl dichloromethane. Sample (1.0 μl) was injected onto a 30 m HP-5 column (J & W Scientific) and analysed using an Agilent 6890N GC-MS. Individual FAMEs were identified according to retention times and mass spectra of standards, and quantified by comparison of peak areas with that of the internal standard.

Site-directed mutagenesis

Five pairs of complimentary primers were synthesized to mutate selected amino acids from $TbPLA_1$ to alanine and they are the following: D63A, 5′-GAGGGACTTAAAAAAAGGGCCGAGAGTTGCGG-3′ and 5′-CCGCAACTCTCGGCCCTTTTTTTAAGTCCCTC-3′; D183A, 5′-GCCGAAGCGCTTGCTGTGTTCCAACGACATCCG-3′ and 5′-CGGATGTCGTTGGAACACAGCAAGCGCTTCGGC-3′; D232A 5′-CAGGCCACATTATATTGCTGCTCACCTCATAGAGAC-3′ and 5′-GTCTCTATGAGGTGAGCAGCAATATAATGTGGCCTG-3′; H234A, 5′-CATTATATTGATGCTGCCCTCATAGAGACAATTATTG-3′ and 5′-CAATAATTGTCTCTATGAGGGCAGCATCAATATA ATG-3′; S131A, 5′-GTTTCATTCACGGGGCATGCGCTTGGCGGTGGTTTG-3′ and 5′-CAAACCACCGCCAAGCGcATGCCCCGTGAATGAAAC-3′. The primers and the vector template, *pET15b/TbPLA*$_1$, were used with the QuickChange Site-directed Mutagenesis kit (Stratagene). Plasmids harbouring the altered *TbPLA*$_1$ were sequenced to confirm the mutation.

Creation of *TbPLA*$_1$ mutants

The 5′- and 3′-UTR sequences immediately upstream and downstream from the *TbPLA*$_1$ gene, respectively, were obtained from the database. 5′-UTR (270 bp) and 3′-UTR (410 bp) fragments were amplified from genomic DNA using the 5′-UTR sense primer (5′-ataagaatgcggccgcTCACCATTTGGCACGTGAGGGA-3′) and antisense primer (5′-cgtttaaacttacggaccgtcaagcttGATTCCTTATAAAATGATTCCACTTG-3′), and the 3′-UTR sense primer (5′-tgacggtccgtaagtttaaacggatccACTCATAACGCAAAA-3′) and antisense primer (5′-gtaagcggccgcTCACAAACAAATATTACAGACAGGAAT-3′). These two products, with 5′-NotI sites, were linked (via the complimentary underlined primer sequence) in a linking PCR reaction, amplified by PCR, NotI digested and ligated into the NotI site of *pGEM5Zf* (Promega). The linker region was excised by HindIII/BamHI digestion and the puromycin (*puro*^{R}) and blasticidin (*bsd*^{R}) drug resistance genes with

HindIII/BamHI sticky ends were ligated individually into the cloning site. The resultant plasmids were NotI digested to release their *5′-puro^R-3′* and *5′-bsd^R-3′* DNA inserts, precipitated with ethanol, and redissolved in sterile water prior to being used in the electroporation and selection of *T. brucei* transgenic populations, performed as described elsewhere (Martin and Smith, 2006a,b).

Ectopic copies of *TbPLA$_1$* were engineered into *pLEW100/PLA1-myc* by inserting the ORF of *TbPLA$_1$* into the HindIII/PacI cloning site of *pLEW100* (Wirtz *et al.*, 1999) by using the HindIII-containing ORF forward (5′-atagtacaagcttATGTCGGAATCCGTTGATCAGT-3′) and PacI-containing reverse primers (5′-gatagcttaattaaTGAAGAACCGAACCCTCCG-3′). Mid-log cells were electroporated with 10 μg of NotI-linearized *pLEW100/PLA1-myc* plasmids in a total volume of 450 μl of cytomix buffer. The transfected parasites were selected in medium containing phleomycin (2.5 μg ml^{-1}). When tetracycline was added to the media during biochemical analysis, a final concentration of 1 μg ml^{-1} was used. Cells were counted each day and were passaged only when the density was between 2 × 10^6 and 3 × 10^6 (normally every second day).

EGFP fused to TbPLA$_1$ was engineered in both a C- and N-terminal orientation to produce the plasmids *pLEW100/EGFP-TbPLA1-myc* (N-terminal) and *pLEW100/TbPLA1–EGFP* (C-terminal). N-terminal *EGFP* was cloned into the HindIII cloning site of *pLEW100/PLA1-myc* using HindIII-containing forward (5′-tagtacaagcttATGGTGAGCAAGGGCGAGG-3′) and reverse (5′-tagtacaagcttCTTGTACAGCTCGTCCATGCC-3′) primers. C-terminal *EGFP* was cloned into the PacI/BamHI cloning site (displacing the *myc* tag) of *pLEW100/PLA1-myc* using PacI-containing forward (5′-gatagcttaattaacATGGTGAGCAAGGGCGAGG-3′) and BamHI-containing reverse (5′-atagtacggatccaTTACTTGTACAGCTCGTCCATGCC-3′) primers. The fusion proteins were tetracycline induced overnight, washed twice in PBS, and fixed in 4% paraformaldehyde. The cells were mounted on polysine slides, washed and stained with DAPI before visualizing EGFP with exposure to UV light on a Zeiss Axiovert 200 imaging microscope.

Southern and Northern blotting

For Southern blotting, *T. brucei* genomic DNA (2 μg/lane) was digested with EcoRI and the fragments separated on a 0.7% agarose gel, transferred to nitrocellulose and UV-cross-linked. For Northern blotting, total RNA was extracted with an RNA Mini Kit (Qiagen) and 12 μg was separated on 1% MOPS/Formaldehyde, 0.7% agarose gel before transfer and linking to nitrocellulose. The blots were blocked with ULTRAhyb™ (Ambion) for 1 h at 42°C before hybridization with fluorescein-labelled *TbPLA$_1$* (southern) or [^{32}P]-CTP-labelled *TbPLA$_1$* probe (northern) overnight at 42°C. The blots were washed twice with 2× SSC plus 0.1% SDS for 20 min each, and washed twice with 0.5× SSC plus 0.1% SDS for 20 min each at 42°C. Northern blots were directly exposed to film whereas bound probe on the Southern blots were detected using horseradish peroxidase-conjugated antifluorescein antibody with the Gene Images CDP-Star kit (Amersham Biosciences). The bands on the Northern blot-transferred images were analysed by densitometry using AIDA.

Electrospray tandem mass spectrometry

Total lipids from 10^8 mid-log PCF trypanosomes were extracted by the method of Bligh and Dyer (1959) and the lower chloroform phase was washed once with fresh upper phase. The resultant lower phase lipid extract was dried under nitrogen and redissolved in 20 μl of chloroform/methanol (1:2). For samples containing internal standards, lysoGPCho(17:0/−) and lysoGPCho(24:0/−) (0.5 nmol/standard) were added prior to lipid extraction. An aliquot of the samples was analysed with a Micromass Quattro Ultima triple quadrupole mass

spectrometer equipped with a nano-electrospray source. Samples were loaded into thin-wall nanoflow capillary tips (Waters) and analysed by ESI-MS-MS in both positive (GPCho, GPSer and GPEth) and negative ion mode (GPIns, GPSer and GPEth) under capillary/cone voltages of 0.7 kV/50 V and 0.9 kV/50 V respectively. Tandem mass spectra were obtained using argon as the collision gas (~3.0 mTorr) with collision offset energies as follows: 35 V, positive ion precursors of *m/z* 184 for GPCho; 45 V, negative ion precursors of *m/z* 241 for GPIns; 25 V, neutral loss scanning of *m/z* 141 in positive mode for GPEth; 22 V, neutral loss scanning of *m/z* 185 in positive mode for GPSer; 50 V, negative ion precursors of *m/z* 196 for GPEth; and 28 V, neutral loss scanning of *m/z* 87 for GPSer. Each spectrum encompasses at least 50 repetitive scans of 4 s duration.

Subcellular fractionation and Western blotting

Log phase bloodstream or PCF form parasites [strain 427, MITat 1.2, cultured as previously described in SDM-79 media at pH 7.4 which contained 15% heat-inactivated fetal calf serum as a lipid source (Martin and Smith, 2006b)] were osmotically lysed at 10^6 cell equivalents ml^{-1} using cold H_2O containing 2 μg ml^{-1} leupeptin and 0.1 mM TLCK. Cell lysis was confirmed by microscopy. Lysate was centrifuged at 1000 *g* to pellet nuclei and unbroken cells. The resultant supernatant was then centrifuged at 14 500 *g* for 10 min to pellet the large granular fraction, which was resuspended with PBS to an equivalent volume of aspirated supernatant. The 14 500 supernatant was centrifuged again at 140 000 *g* for 1 h. The resultant pellet, the small granular fraction, was resuspended in PBS after aspirating the supernatant, the cytosolic protein fraction. The proteins from 10 μl of each fraction were resolved on a 15% SDS-PAGE gel and analysed by Western blot. The blot was developed using an ECL detection kit according to the manufacturers' instructions (Amersham Biosciences). Purified recombinant $TbPLA_1$ was used to obtain rabbit antiserum (Eurogentec). Anti-rabbit $TbPLA_1$ antibodies were affinity purified using a recombinant $TbPLA_1$-coupled CNBr-activated Sepharose column and following the protocol outlined by the manufacture (Amersham Biosciences). If needed, the bands on the Western blottransferred images were analysed by densitometry using AIDA.

Bis-BODIPY® FL-C_{11}-PC assay

Bis-BODIPY® FL-C_{11}-PC (Molecular Probes) was used as a GPCho fluorescent substrate at a 1:100 molar ratio with GPCho(16:0/16:0), and TX-100/lipid micelles were made. In its diacyl state, the proximity of the BODIPY fluorophores on adjacent PL acyl chains causes an intramolecular self-quenching of fluorescence. Hydrolysis and release of an acyl chain by a phospholipase A alleviates quenching and an increase in fluorescence results. The fluorophores are situated at the ends of two 11 carbon chains thus mimicking a GPCho with an approximate length near to that of GPCho(16:0/16:0).

The assay was developed for use in a 96 well plate. The assay was performed at a mole fraction of 0.018 [mole fraction = (lipid)/(lipid + detergent)]. Micelles were made as an 8× working solution which was then diluted accordingly for use in an 80 μl reaction per well. Each well initially contained 40 μl of buffer (50 mM Tris-HCl, pH 7.0) containing the micelle substrate (0.075 mM final). The reaction was started by the addition of 40 μl reaction buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.0) containing 400 pg (11.4 pmol) of recombinant $TbPLA_1$. The reaction was left to proceed at 22°C for 10 min before quenching with 170 μl of 100% methanol. Liberated acyl chains containing a fluorophore were excited at 485 nm and detected through a 520 nm centred bandpass filter using the FLX 800 Microplate Reader (Bio-Tek Instruments). All experiments were performed at least three times in triplicate and data points varied by less than one standard deviation. Velocities obtained were initial velocities.

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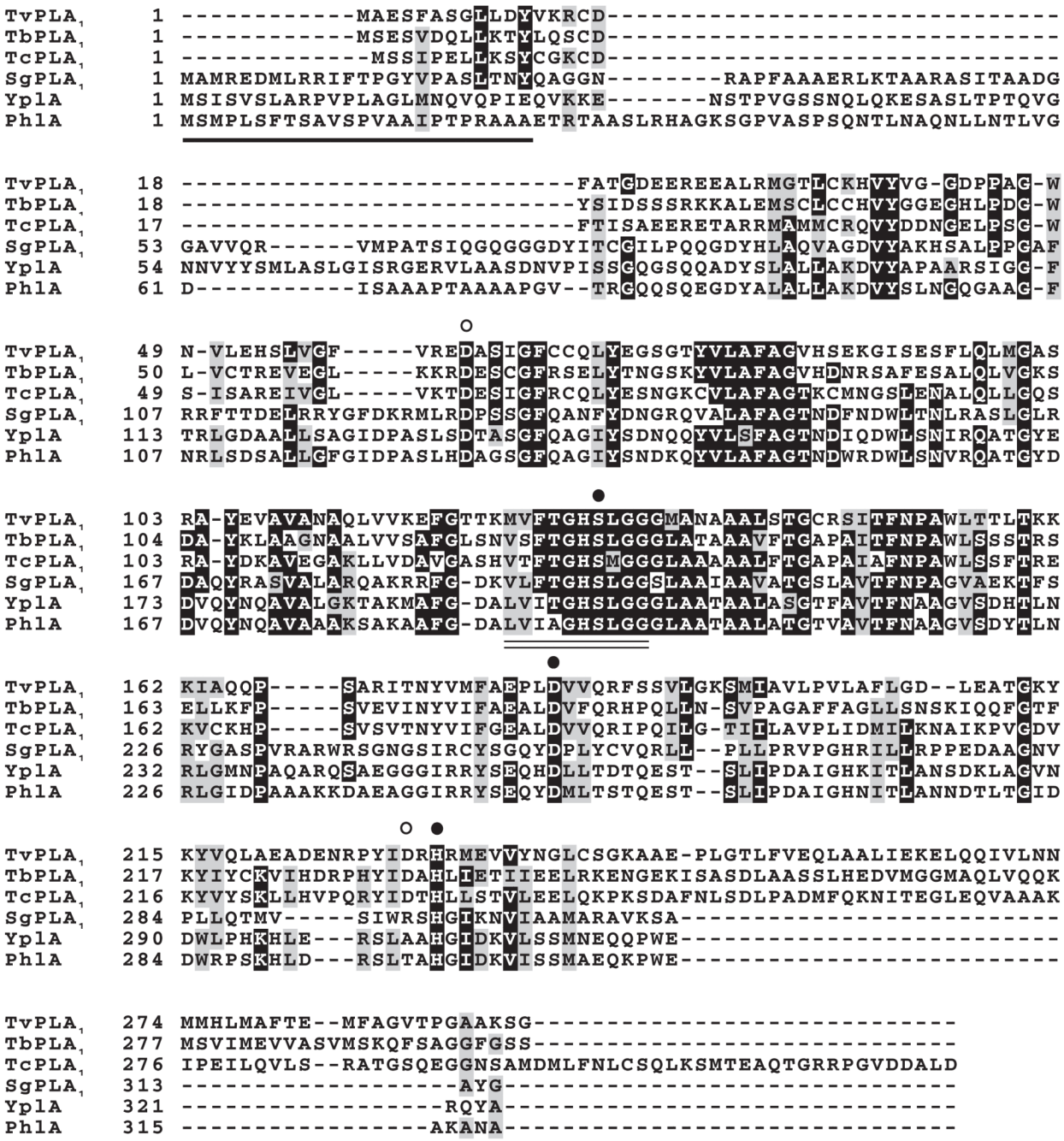

Fig. 1.

Multiple sequence alignment of trypanosome and bacterial PLA_1. Amino acid sequences were aligned with MUSCLE (Edgar, 2004) and edited using the BOXSHADE algorithm. Amino acid residues below circles represent those which were mutated to reveal active-site components (closed circles) and non-active-site residues (open circles). The lipase consensus pattern is underscored by a double line and the signal sequence utilized for PhlA secretion (Givskov *et al.*, 1988) is underlined. $TcPLA_1$, *Trypanosoma congolense* PLA_1 (product of GeneDB Systematic name congo50h08.q1k_9); $TbPLA_1$, *Trypanosoma brucei* PLA_1 (Accession Number CAG29794); $TvPLA_1$, *Trypanosoma vivax* PLA_1 (product of GeneDB Systematic name Tviv1355g06.p1k_2); $SgPLA_1$, *Sodalis glossinidius* PLA_1

(Accession Number Q2NQT2); YplA, *Yersinia enterocolitica* PLA_1 (Accession Number O85477); PhlA, *Serratia liquefaciens* PLA_1 (Accession Number P18952).

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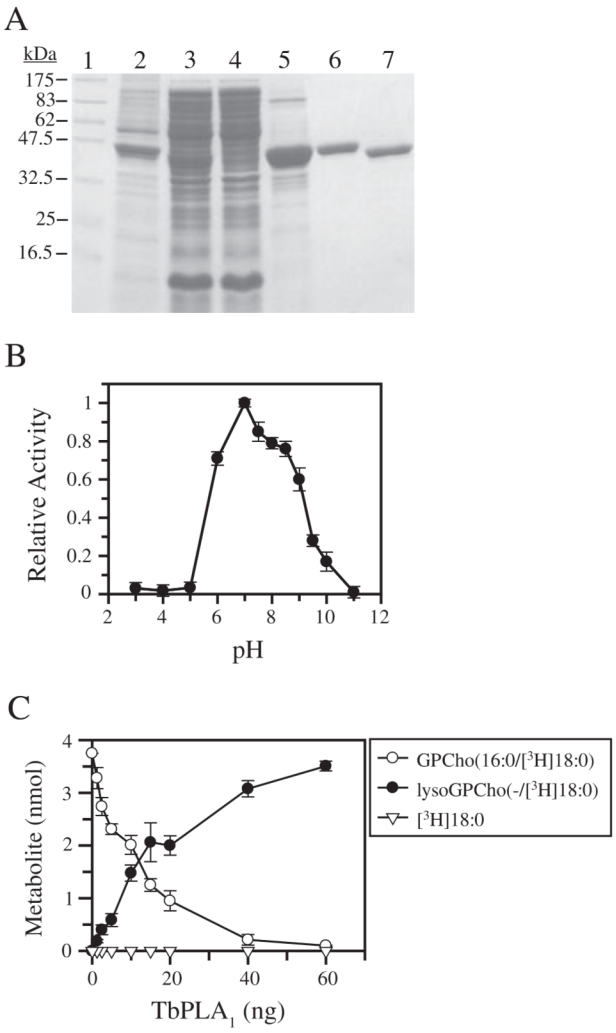

Fig. 2.

Purification and regiospecificity of recombinant $TbPLA_1$.

A. SDS-PAGE analysis of proteins from $TbPLA_1$-overexpressing *E. coli* cells during various purification steps as compared with molecular weight standards (lane 1). The various fractions include insoluble proteins (lane 2), the soluble protein fraction containing His·tagged recombinant $TbPLA_1$ (lane 3), nickel column flow through (lane 4), nickel affinity eluate (lane 5), purified His·tagged recombinant $TbPLA_1$ after anion exchange (~3 μg) (lane 6), purified His·tag-cleaved $TbPLA_1$ after thrombin digestion and anion exchange (~3 μg) (lane 7).

B. Phospholipase activity was measured at various pH values using the fluorescent BODIPY®C_{11}-PC assay at a mole fraction of 0.018 with a final concentration of substrate at 0.075 mM. A universal buffer containing 50 mM Tri-sodium citrate, 50 mM Tris and 50 mM NaCl was used to obtain the various pH conditions. Data points represent the activity measured relative to the maximal pH-activity value of pH 7.0. Data represent the average from two experiments performed in triplicate.

C. Metabolism of GPCho(16:0/[^{3}H]18:0) after a 5 min reaction is shown as a function of varying amounts of recombinant $TbPLA_1$. Formation of lysoGPCho(−/[^{3}H]18:0) appears to be dose-dependent, whereas [^{3}H]18:0 acid cleavage from the *sn*-2 position is undetectable.

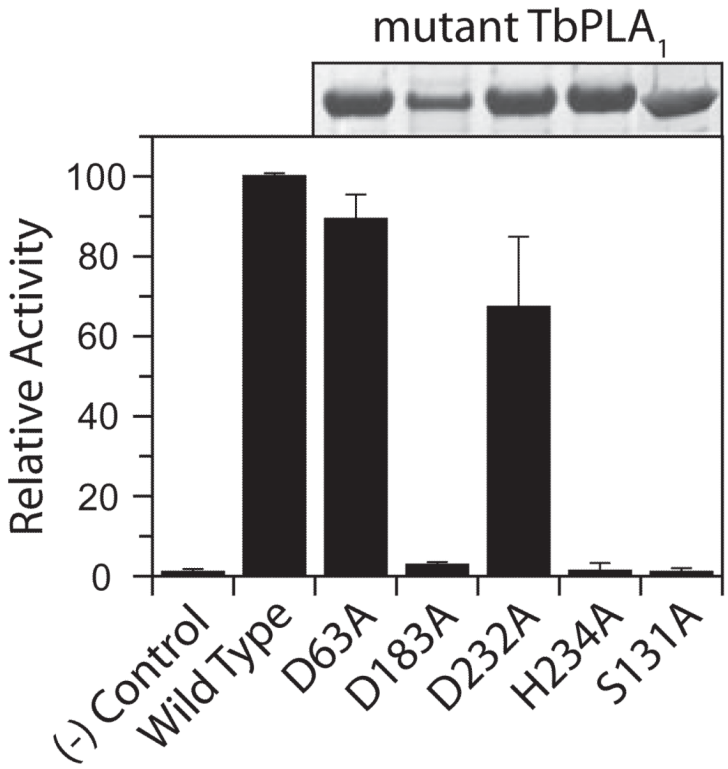

Fig. 3.

Probing for active-site residues. Mutant $TbPLA_1$ proteins were expressed and purified from *E. coli* in parallel as stated in the *Experimental procedures* and 10 μl of the final eluate was analysed by SDS-PAGE (above graph). The radiolabelled PLA_1 assay was used with 5 ng of purified WT and mutant $TbPLA_1$ to compare substrate hydrolysis after 10 min; a no-enzyme negative control was tested in parallel. Mutant activities are shown as relative percentages of WT activity (26 μmol min^{-1} mg^{-1}).

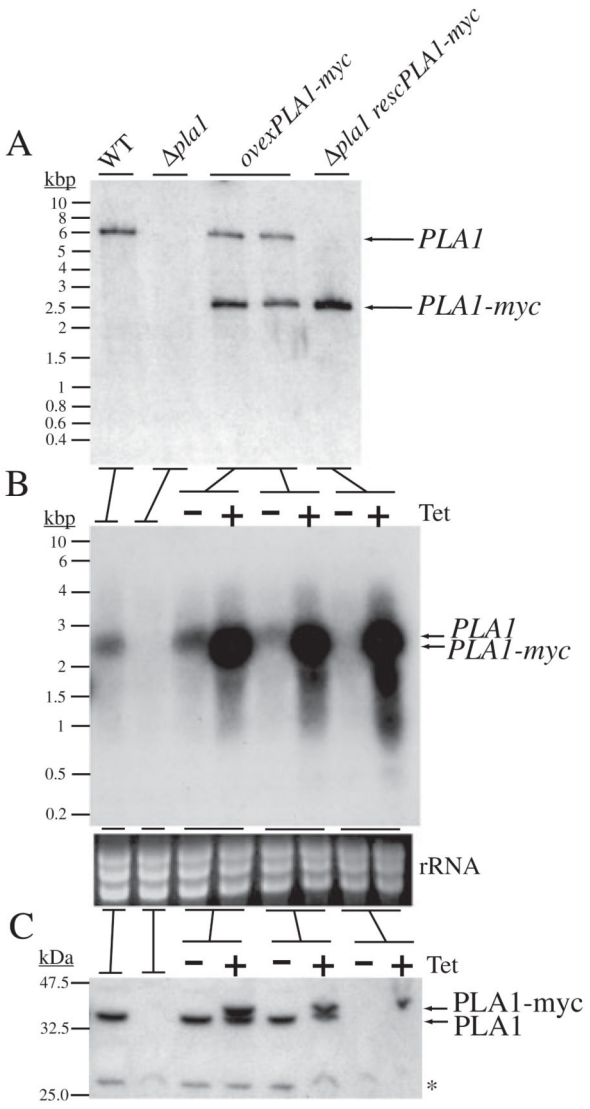

Fig. 4.

Validation and characterization of *TbPLA*$_1$ mutant cell lines.

A. *TbPLA*$_1$ null mutants were generated in the procyclic form of *T. brucei* after sequential *TbPLA*$_1$ UTR-targeted homologous recombination with UTR-flanked puromycin and blasticidin resistance genes. Cell lines were analysed by Southern blot after digesting genomic DNA with EcoRV. EcoRV-digested *T. brucei* WT gDNA reveals one size of DNA fragment detectable by fluorescein-labelled *TbPLA*$_1$ ORF probe. In *TbPLA*$_1$ null mutant cells (Δ*pla1*) both *TbPLA*$_1$ alleles are absent. A tetracycline (Tet)-inducible *myc*-tagged recombinant ectopic copy of *TbPLA*$_1$ (*PLA1-myc*) cloned into a phleomycin-resistant *pLEW100* cassette was introduced into a different locus in WT gDNA to produce transgenic *TbPLA*$_1$ overexpression cell lines (*ovexPLA1-myc*), or introduced in the null mutant gDNA to produce a rescue cell line (Δ*pla1 rescPLA1-myc*).

B. Northern blot analysis of *TbPLA*$_1$ mutants. Total RNA extracted from WT and *TbPLA*$_1$ mutant trypanosomes were hybridized with *TbPLA*$_1$ (top panels), loading controls are also presented (bottom panels).

C. Western blot analysis of cell lysates of *TbPLA*$_1$ mutants. Both native PLA$_1$ (theoretically 32.4 kDa) and tagged PLA1-myc (theoretically 33.9 kDa) proteins were detected by

antibodies raised against the purified recombinant enzyme. * = background bands that show loading in the null mutant lanes.

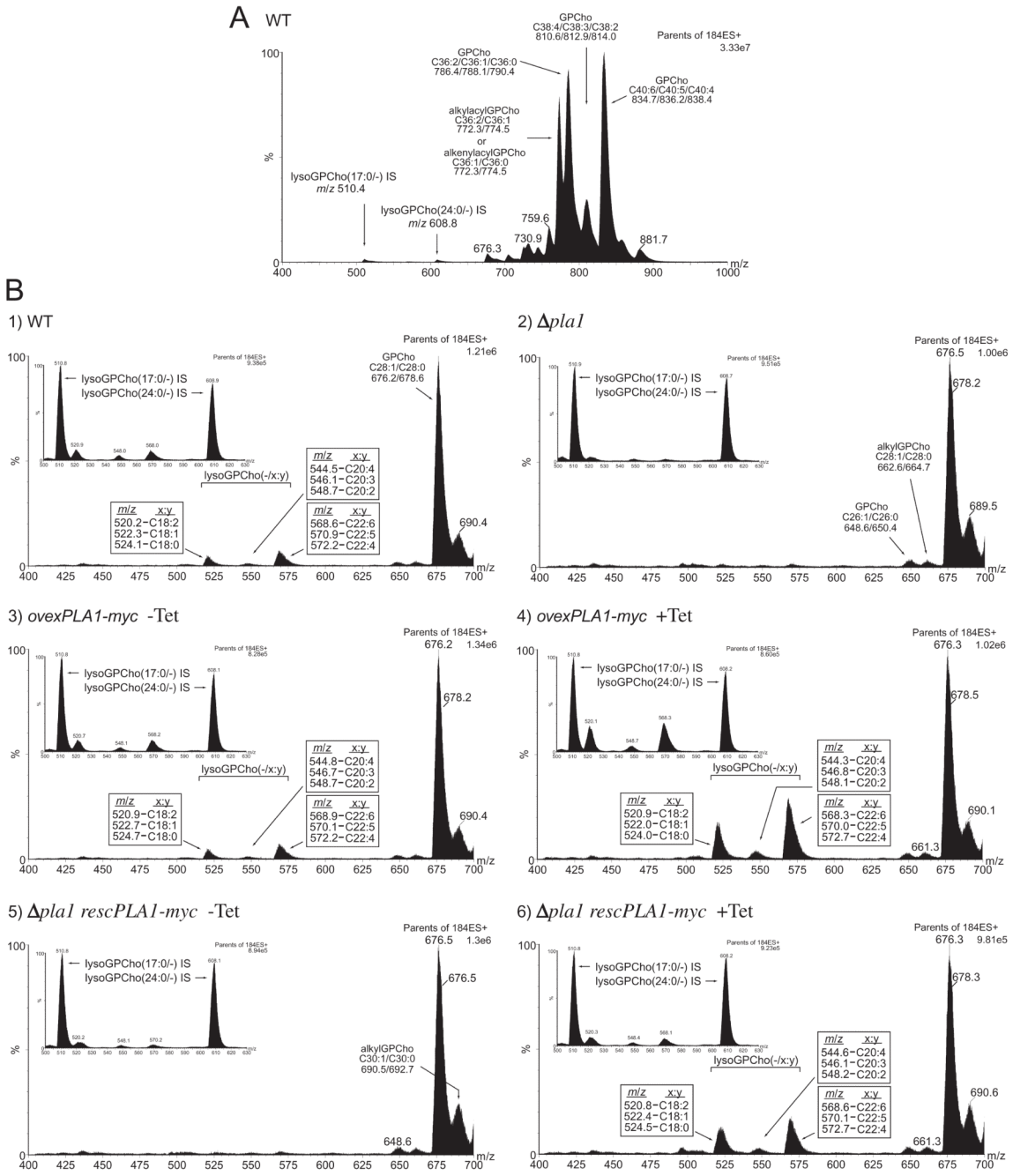

Fig. 5.

TbPLA$_1$ functions to synthesize long-chain unsaturated lysoGPCho intermediates.

A. Positive ion ESI-MS-MS spectrum of *m/z* 184 lipid precursors in total lipid extracts from *T. brucei* WT cells. Identities of the major $[M+H]^+$ ions are indicated as well as internal standard lysoGPCho $[M+H]^+$ peaks.

B. Positive ion ESI-MS-MS short-range spectra of the parents of the *m/z* 184 ion from total lipid extracts from WT and *TbPLA*$_1$ mutant cell lines. The major sets of lysoGPCho $[M+H]^+$ metabolites detected are boxed and annotated next to the *m/z* peak from which they are derived. The numbers in place of *x*:y refer to the total number of *sn*-2 FA carbon atoms (x) and their degree of unsaturation (y). Panel insets are similar short-range spectra from

lipid extracts spiked with lysoGPCho internal standards used for quantitative purposes (Table 2).

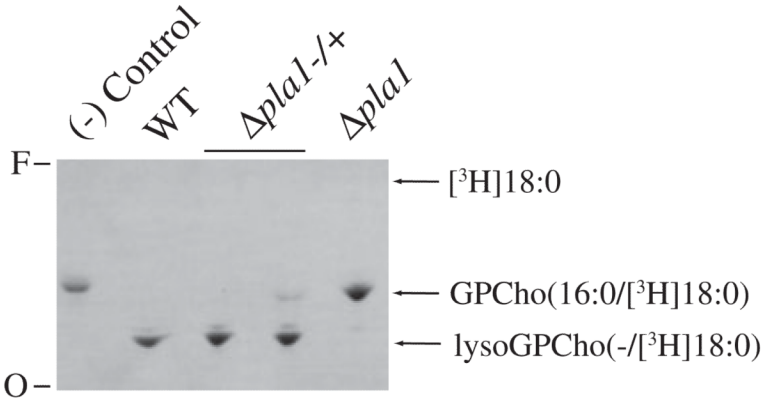

Fig. 6.

Phospholipase activity from lysates of *T. brucei* cells. PCF phospholipase activity from 10^6 cell equivalents was examined after 20 min incubation with GPCho(16:0/[^{3}H]18:0). The TLC autoradiograph shows the metabolism of GPCho(16:0/[^{3}H]18:0) into lysoGPCho(−/[^{3}H]18:0) by PLA_1 in WT and single knockout (Δ*pla1*−/+) lysate. PLA_1 activity is absent in the cell-free control and the Δ*pla1* double knockout cell line. To stimulate any possible PLA_2 activity in the lysates 10 mM $CaCl_2$ was added to each assay. Even with the dominant PLA_1 activity being absent from Δ*pla1* clones, no PLA_2 activity was observed in their lysate. Free [^{3}H]18:0 was used as a standard to monitor PLA_2 activity and its R_f is indicated. O, origin; F, front.

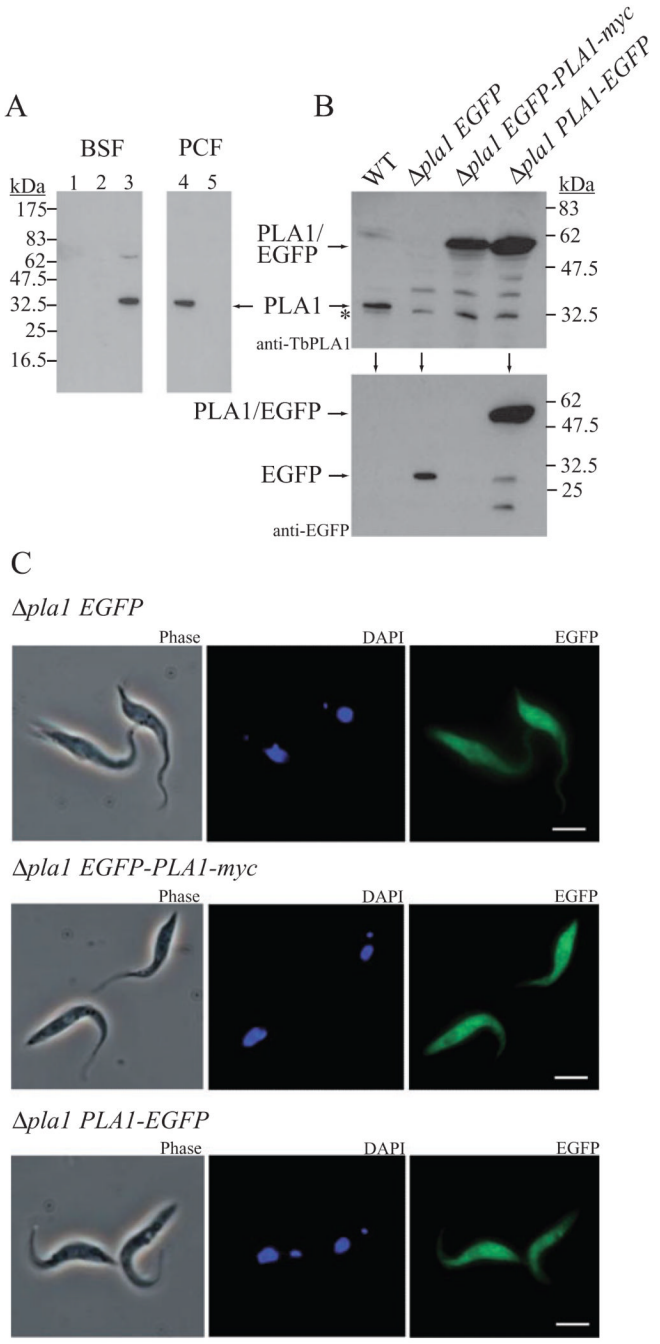

Fig. 7.

Cellular distribution of $TbPLA_1$.

A. Subcellular fractions were prepared as described in *Experimental procedures* and used in immunoblotting with anti-$TbPLA_1$ antibodies. $TbPLA_1$ is constitutively expressed and was detected in the cytosolic fraction of both BSF and PCF WT cells (lane 3 and lane 4 respectively) but absent from PCF *$TbPLA_1$* null mutants (lane 5). $TbPLA_1$ is also absent from the large granular and small granular (microsomal) fractions from BSF WT cells (lane 1 and lane 2 respectively).

B. PCF *$TbPLA_1$* null mutant cells transformed with an ectopic copy of an N-terminal *EGFP-PLA1* gene fusion (Δ*pla1 EGFP-PLA1-myc*) or a C-terminal *PLA1-EGFP* gene fusion

(Δ*pla1 PLA1-EGFP*) were analysed after tetracycline induction by anti-TbPLA$_1$ (top panel) or anti-EGFP (lower panel) immunoblotting for the presence of expressed fusion protein (~60 kDa). WT cells and null mutants transformed with an ectopic copy of *EGFP* only (Δ*pla1 EGFP*) serve as negative controls for fusion protein expression. * = background bands just smaller than native TbPLA$_1$ that show loading in the null mutant lane.
C. Analysis of the subcellular localization by immunofluorescence microscopy of both PLA1/EGFP fusion proteins (right panels) compared with EGFP only confirms a cytoplasmic distribution for soluble TbPLA$_1$. PCF transgenic mutants are also shown in phase contrast (left panels) and after DAPI staining (middle panels).

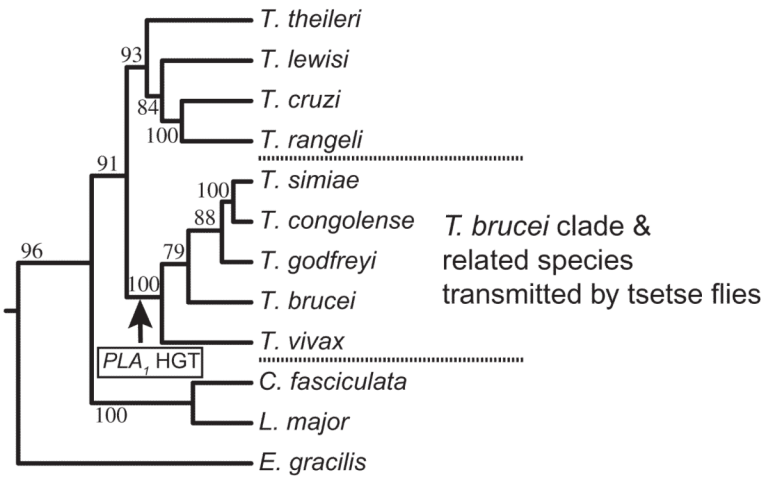

Fig. 8.

Proposed HGT point for *S. glossinidius* PLA_1. The arrow indicates the proposed acquisition of *S. glossinidius PLA*$_1$ by an ancestor of *T. brucei*. The maximum likelihood tree was calculated using the Phylip v3.6 Dnamlk algorithm and based on a MUSCLE alignment of glycosomal glyceraldehyde 3-phosphate dehydrogenase (*gGAPDH*) genes from various kinetoplastids. Values at nodes are maximum likelihood bootstrap values (%) obtained from 100 replicates (Seqboot). The tree is rooted to the corresponding gene of *Euglena gracilis*. The tree phylogenies agree very well with the more detailed and exact *gGAPDH* trees previously published (Hamilton *et al.*, 2004; 2005). The *gGAPDH* sequences were obtained from GenBank and their accession numbers are: *Trypanosoma theileri*, AJ620282; *Trypanosoma lewisi*, AJ620272; *Trypanosoma cruzi*, AJ620269; *Trypanosoma rangeli*, AF053742; *Trypanosoma simiae*, AJ620293; *Trypanosoma congolense*, AJ620291; *Trypanosoma godfreyi*, AJ620292; *Trypanosoma brucei*, AJ620284; *Trypanosoma vivax*, AJ620295; *Crithidia fasciculata*, AF047493; *Leishmania major*, AF047497; *Euglena gracilis*, L21903.

Table 1

Hydrolysis of lipid substrates by $TbPLA_1$

Substrate	PLA_1 activity[a] (μmol min^{-1} mg^{-1}
GPCho(16:0/18:1)	120.2 ± 18.7
GPCho(18:0/20:4)	118.4 ± 5.4
GPCho(16:0/14:0)	114.3 ± 6.0
GPCho(14:0/18:0)	110.9 ± 2.0
GPCho(14:0/14:0)[b]	100.4 ± 8.7
GPCho(16:0/16:0)[b]	84.8 ± 7.4
GPCho(16:0/18:0)	78.5 ± 7.9
GPCho(18:0/18:0)[b]	54.0 ± 7.6
GPSer(16:0/16:0)[b]	49.2 ± 7.6
lysoGPCho(14:0/−)	16.1 ± 1.0
GPA(16:0/16:0)[b]	8.6 ± 0.7
GPEth(16:0/16:0)[b]	7.3 ± 2.4
Bovine liver GPIns[b,c]	0.7 ± 0.04[d]
DG(16:0/16:0)[b]	0.5 ± 0.06[d]
TG(18:0/18:0/18:0)	ND[d]
GPCho(1-0-16:0/16:0)	ND

[a] Assays were conducted as described under *Experimental procedures*. Activity is expressed as micromoles of *sn*-1 FA released per min mg^{-1} of protein. Values are means ± SD, $n = 3$ determinations, each performed in triplicate. ND, none detected.

[b] Regiospecificity of FA hydrolysis detected cannot be discerned with this substrate.

[c] The only FAME detected was that from stearic acid (C18:0), which comprises nearly 50% of the FAs in bovine liver GPIns.

[d] 1.85 μg of enzyme was added instead of 0.1 μg, and the reaction was extended to 20 min. Less than 10% of substrate was turned over.

Table 2

Quantitative analysis of lysoGPCho metabolites in *TbPLA*$_1$ mutants.[a]

		lysoGPCho series[b]			
Cell line	**Tet**	**(−/18:y)**	**(−/20:y)**	**(−/22:y)**	**Total**
WT		53	31	72	156
Δ *pla1*		15	8	7	30
ovexPLA1-myc	−	56	22	81	159
	+	159	42	231	432
Δ *plal rescPLAI-myc*	−	29	14	23	66
	+	52	22	54	128

[a]Total lipid extracts from 10^8 PCF *T. brucei* cells spiked with 500 pmol of each internal standard [lysoGPCho(17:0/−) and lysoGPCho(24:0/−)] were examined by ESI-MS-MS. lysoGPCho molecules were detected from precursor ion scanning for *m/z* 184 (see insets from Fig. 5B), and they were quantified by comparison with the internal standards. Values are from a typical analysis and are presented as pmol per 10^8 cells. Tet, tetracycline.

[b]Integration of peaks to obtain peak areas was performed by grouping lysoGPCho into various series of peaks whereby one series comprises both the major and minor lysoGPCho molecules, and their isotopes, containing the same FA chain length but with varying degrees of unsaturation, represented by ‘y’.