The role and characterization of phospholipase A₁ in mediating lysophosphatidylcholine synthesis in *Trypanosoma brucei*

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Lysophospholipids are ubiquitous intermediates in a variety of metabolic and signalling pathways in eukaryotic cells. We have reported recently that lysoglycerophosphatidylcholine (lyso-GPCho) synthesis in the insect form of the ancient eukaryote *Trypanosoma brucei* is mediated by a novel phospholipase A₁ (TbPLA₁). In the present study, we show that despite equal levels of TbPLA₁ gene expression in wild-type insect and bloodstream trypomastigotes, both TbPLA₁ enzyme levels and lysoGPCho metabolites are approx. 3-fold higher in the bloodstream form. Both of these parasite stages synthesize identical molecular species of lysoGPCho. TbPLA₁ null mutants in the bloodstream form of the parasite are viable, but are deficient in lysoGPCho synthesis, a defect that can be overcome by the expression of an ectopic copy of TbPLA₁. The biochemical attributes of TbPLA₁-

mediated lysoGPCho synthesis were examined *in vitro* using recombinant TbPLA₁. Although TbPLA₁ possesses an active-site serine residue, it is insensitive to serine-modifying reagents, such as di-isopropyl fluorophosphate and PMSF, a characteristic shared by lipases that possess lid-sheltered catalytic triads. TbPLA₁ does not require metal co-factors for activity, but it does require interfacial activation prior to catalysis. Results from size-exclusion chromatography and binding kinetics analysis revealed that TbPLA₁ activation by Triton X-100/GPCho mixed micelle surfaces was not specific and did not require the pre-formation of a specific enzyme–substrate complex to achieve surface binding.

Key words: interfacial activation, lipid metabolism, lysophosphatidylcholine, phospholipase, *Trypanosoma brucei*.

INTRODUCTION

All eukaryotic organisms are thought to metabolize phospholipids by expressing a highly regulated and variable complement of the phospholipases A₁, A₂, C, D and B. In *Trypanosoma brucei*, a medically important protist that causes African sleeping sickness, the complement of cellular phospholipases has not been fully elucidated. The only identified phospholipase in *T. brucei* was GPI-PLC (glycosylphosphatidylinositol-phospholipase C), which specifically recognizes GPI membrane anchors [1–3]. However, a highly active PLA₁ has recently been cloned in *T. brucei* [4]. TbPLA₁ (*T. brucei* PLA₁) exhibits substrate preference towards GPCho (glycerophosphatidylcholine) and produces unsaturated lysoGPCho (lysoglycerophosphatidylcholine) metabolites *in vivo*, approx. 50 % of which contain a long and highly unsaturated FA (fatty acid) chain, consisting of 22 carbon atoms [4]. Bacteria secrete PLA₁ [5,6], but TbPLA₁, which has a bacterial origin, is localized to the cytosol of *T. brucei* [4]. In the insect PCF (procyclic form) of *T. brucei*, TbPLA₁-synthesized lysoGPCho metabolites have homeostatic levels of approx. 156 pmol/10⁸ cells [4].

LysoGPCho is an amphiphilic lipid metabolite derived from GPCho, whose levels are vitally managed by balancing synthesis with degradation. As in other eukaryotes, GPCho is the most abundant phospholipid in *T. brucei*, comprising approx. 57 % and 48 % of total cellular phospholipid in the PCF and the mammalian BSF (bloodstream form) respectively [7,8]. Unlike in other eukaryotes, however, how *T. brucei* cells regulate GPCho homeostasis is not understood. In higher eukaryotes, GPCho is metabolized by one of a number of lipolytic reactions, the best characterized ones being the following: 1) saturated lysoGPCho is synthesized by a PLA₂,

of which one type deacylates GPCho-derived arachidonic acid for use in cell signalling [9]; 2) saturated lysoGPCho is synthesized by the action of LCAT (lecithin–cholesterol acyltransferase) [10]; 3) PLD catalyses the hydrolysis of GPCho to phosphatidic acid and choline [11]; or 4) PLB deacylates the fatty acyl moieties of GPCho to form glycerophosphocholine and free FAs [12–14], a degradation pathway which is stimulated by Sec14 [15,16]. Interestingly, *T. brucei* is thought to lack PLA₂ activity [17] and analysis of its genome does not reveal putative PLA₂ or PLD homologues [4]. On the other hand, several LysoPLA (lysophospholipase A)/PLB homologues are present in the database, which may explain previous studies that reported their corresponding specific esterase activity [18–21]. Despite the possession of an LCAT homologue in *T. brucei*, this parasite contains minute quantities of saturated lysoGPCho species and instead synthesizes mostly polyunsaturated and highly unsaturated lysoGPCho by TbPLA₁ [4].

T. brucei spends much of its life in the mammalian systemic circulation from which it acquires its nutrients, including saturated and unsaturated lysoGPCho [18,22]. Unsaturated lysoGPCho in mammalian plasma accounts for roughly only 1–4 % of total plasma phospholipids [23] and, although it is formed by an undefined mechanism, it is derived from hepatic secretions [24]. It was originally thought that plasma lysoGPCho acquired by the BSF of *T. brucei* was detoxified by the cell's robust PLA₁ activity [18,19,25]. This is most probably not the case, however, since TbPLA₁ is cytosolic and metabolizes endogenous GPCho [4]. With a long-term goal to elucidate the biological significance of TbPLA₁ in *T. brucei*, in the present study, we investigate endogenous lysoGPCho synthesis in the BSF of *T. brucei* and relate this

Abbreviations used: [M + H]⁺, mass plus hydrogen positive ion; BODIPY®, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; BSF, bloodstream form; EA, enzyme-aggregate; ESI-MS/MS, electrospray ionization tandem MS; FA, fatty acid; GPCho, glycerophosphatidylcholine; GPI, glycosylphosphatidylinositol; HA, haemagglutinin; hyg^R, hygromycin resistance; iPr₂P-F, di-isopropyl fluorophosphate; LCAT, lecithin–cholesterol acyltransferase; lysoGPCho, lysoglycerophosphatidylcholine; ORF, open reading frame; PCF, procyclic form; puro^R, puromycin resistance; TbPLA₁, *Trypanosoma brucei* phospholipase A₁; TetR, tetracycline repressor; UTR, untranslated region; WT, wild-type.

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activity to its insect-stage equivalent. We also characterize further *TbPLA₁*-mediated lysoGPCho synthesis *in vitro* in order to further understand the properties of this novel eukaryotic enzyme.

EXPERIMENTAL

Materials

Analytical reagents were bought from Sigma unless stated otherwise. Synthetic phospholipids were purchased from Avanti Polar Lipids. Chemicals used for bulk buffer production were from BDH unless stated otherwise. All solvents were of HPLC grade and purchased from BDH.

Generation and analysis of BSF *TbPLA₁* mutants

Deletion constructs for BSF WT (wild-type) transformations were synthesized by a series of amplification and cloning steps similar to the methods used to obtain PCF *TbPLA₁* mutants [4]. Briefly, the 5'-UTR (untranslated region) and 3'-UTR sequences of *TbPLA₁* were amplified, linked together and inserted into a cloning vector. Puromycin (*puro^R*) and hygromycin (*hyg^R*) drug resistance genes were then ligated individually between the UTR sequences, which generated the deletion constructs 5'-*puro^R*-3' and 5'-*hyg^R*-3'. The overexpression vector pLEW82-PLA1-HA was constructed by cloning *TbPLA₁* into pLEW82 using the same primers used to construct pLEW100-*TbPLA₁* [4].

The BSF *in vitro* culture cell line (strain 427, MITat 1.2) used throughout the present study was from the long-term cultures of 'single marker' cells from Wirtz et al. [26] that express a TetR (tetracycline repressor) protein and T7 RNA polymerase, and was maintained under neomycin drug pressure at a final concentration of 2.5 µg/ml. BSF cultures were maintained below a cell density of 2×10^6 /ml in HMI-9 medium at pH 7.5, supplemented with 10% (v/v) heat-inactivated foetal bovine serum (PAA Labs) and 10% (v/v) Serum Plus (JRH Biosciences) as lipid sources. BSF transformation with DNA constructs was achieved through electroporation as described previously [4], except that the cells were left to recover in the incubator for 6–24 h in 24 ml of HMI-9 before distribution and drug selection in a 12-well plate. Transformed cells containing the *puro^R* and/or *hyg^R* genes were cultured in the presence of these drugs at final concentrations of 0.1 µg/ml and 4 µg/ml respectively. Drug resistant parasites appeared between 5 and 7 days, at which time 50 µl of cells was used to start a new culture. Clonal populations of cells were obtained through limiting dilution.

BSF cell lines were analysed by Southern and Northern blotting, performed exactly as described previously [4], as was the nano-ESI–MS/MS (electrospray ionization tandem MS) analysis. Western blot analysis was performed on 10^7 cell equivalents/lane that were obtained by resuspending cultured cells in boiling SDS/PAGE sample buffer at a concentration of 10^6 cells/µl.

Infectivity of genetically modified parasites was examined in mice ($n = 5$ mice for each cell line). Adult mice (BALB/c) were infected intraperitoneally with a single injection of either 5×10^5 WT *T. brucei* bloodstream trypomastigotes or 5×10^5 genetically modified *TbPLA₁* null mutants. Blood parasitaemia was monitored at 24, 48 and 72 h.

ESI–MS/MS

Total lipids from 10^8 mid-log phase BSF trypanosomes were extracted and analysed as described previously [4]. Briefly, the resultant lower phase lipid extract from a Bligh and Dyer extraction [27] was dried under nitrogen and re-dissolved in 20 µl of chloroform/methanol (1:2, v/v). Internal standards (0.5 nmol/standard), lysoGPCho_{17:0/–} and lysoGPCho_{24:0/–}, were added

prior to lipid extraction. Aliquots (7 µl) were analysed by nanoflow capillary ESI–MS/MS in positive-ion mode for GPCho species with a Micromass Quattro Ultima triple quadrupole mass spectrometer with capillary/cone voltages and collision gas as previously described [4]. Each spectrum encompasses at least 50 repetitive scans of 4 s duration.

'Surface dilution' kinetic scheme

For clarity, the concept of 'surface dilution' kinetics is briefly explained here. Lipolytic enzymes act preferentially on substrates that are aggregated at lipid/water interfaces, and this makes kinetic analysis with lipids more complicated than with simple water-soluble substrates. Interface-dependent enzymes work in an environment where bulk interactions occur three-dimensionally in solution, and surface interactions occur in two-dimensions, and any kinetic model for these types of enzymes must consider both types of actions in their proposed mechanism. Deems et al. [28] proposed the 'surface dilution' kinetic model after showing that both the surface concentration of lipid and the bulk concentration of lipid in a reaction play important roles in elucidating kinetic parameters of enzymes that require interfacial activation.

The 'surface dilution' kinetic model's interfacial activation principles are expressed in the following manner:

A soluble enzyme (E) initially associates with a lipid/Triton X-100 mixed micelle aggregate (A) to form an enzyme–aggregate complex (EA). Once the enzyme associates with the aggregate it proceeds to bind to a substrate lipid molecule (B) in its catalytic site thus forming another complex (EAB). Products of the reaction (Q) are formed during catalysis and the EA complex is regenerated. The previous two steps are confined to the surface, and so the reaction is a function of the surface concentration of the substrate and not its bulk concentration, which is a deviation from classic Michaelis–Menton kinetics. Owing to the surface being composed of both lipid and detergent, substrate surface concentration is expressed as a unitless mole fraction, $X_s = [\text{lipid}]/[\text{lipid} + \text{detergent}]$, and it is thus possible to vary the substrate surface concentration simply by adding or subtracting detergent, hence the name 'surface dilution' kinetics.

The initial EA complex can be formed in two ways and 'surface dilution' kinetics can be applied to determine in what form EA exists. It is possible that the enzyme binds non-specifically to the micelle surface, the 'surface binding' model, in which case term A is expressed as the molar concentration of both the lipid and detergent that make up the micelle [28]. Conversely, it is feasible that the enzyme could bind specifically to a lipid substrate in the micelle with one binding site, forming EA, before binding specifically at another site on another lipid molecule for catalysis, which has been termed the 'dual phospholipid binding' model [29,30]. The data analysis performed in the present study used equations derived previously and made the same assumptions necessary to ascertain kinetic parameters [28].

Bis-BODIPY® (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) FL-C₁₁-PC kinetic assay

Bis-BODIPY® FL-C₁₁-PC (Molecular Probes) was used as a GPCho fluorescent substrate at a 1:100 molar ratio with GPCho_{16:0/16:0}, and Triton X-100/lipid micelles were made with this mixture. In its diacyl state, the proximity of the BODIPY® fluorophores on adjacent phospholipid 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. Phospholipid and Triton X-100 varied according to experimental conditions, and the micelles were made as an 8× working solution, which was then diluted accordingly for use in an 80 µl reaction/well. Each well initially contained 40 µl of buffer (50 mM Tris/HCl, pH 7.0) containing the desired amount of micelle substrate. The reaction was started by the addition of 40 µl of reaction buffer (50 mM Tris/HCl, pH 7.0, and 100 mM NaCl) containing 400 pg (11.4 pmol) of recombinant TbPLA₁, obtained as described previously [4]. The reaction was left to proceed at 22 °C for 30 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 an 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 S.D. Velocities obtained were initial velocities. When the assay was used to test activity in the presence of metals and inhibitors, the variable conditions are stated in the legends to the respective Tables. Statistical significance was measured by applying a Student's paired *t* test with a minimum 95 % confidence interval.

Preparation of lipid/detergent mixed micelles

Manufacturer stock vials of phospholipid were dissolved at 2.5–10 mg/ml with chloroform:methanol (1:2, v/v) and stored in the dark at –80 °C until use. The desired amount of the fluorescent substrate mix was transferred into 1.5 ml Apex screw-cap microtubes (Alpha) and dried under nitrogen, while being protected from light. The lipids were resuspended in micelle buffer (50 mM Tris/HCl, pH 7.0, and the appropriate quantity of Triton X-100 to make the desired mole fraction) heated to 40 °C. This solution was then vigorously vortexed for 1 min and placed in a 40 °C sonicator (Ultrawave Limited) until the solution cleared, with intermittent vortexing. The fluorescent mixed micelles were prepared fresh.

Gel-filtration analysis

A Superdex 200 10/300 GL column (Tricorn) with an approximate bed volume of 24 ml was connected to an ÄKTA Purifier pump and equilibrated in a detergent-free buffer (50 mM Tris/HCl, pH 7.5, and 50 mM NaCl) over one and a half column volumes with a flow rate of 0.5 ml/min at 22 °C. The column was calibrated with a mixture of molecular mass markers (Bio-Rad 151-1901). The markers were prepared as instructed by the manufacturer and 150 µl of the standard solution was diluted with buffer to 250 µl, loaded onto the column, separated by size-exclusion chromatography and detected by UV absorbance at 280 nm. The markers and their respective molecular masses are as follows: thyroglobulin, 670 kDa; γ-globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; and vitamin B₁₂, 1.35 kDa. A calibration curve was prepared by measuring the elution volumes of the standards, and then calculating their corresponding partition coefficient (*K_{av}*) values, and plotting their *K_{av}* values versus the logarithm of their molecular mass. *K_{av}* values were calculated from the equation:

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

Figure 1 *TbPLA₁* expression profile

(A) Northern blot analysis of BSF and PCF WT total RNA (12 µg) probed with the *TbPLA₁* ORF (top panel). Constitutive expression is revealed by the detection of a single band in both lanes. Loading was controlled for by re-probing the blot with the *β-tubulin* ORF (bottom panel). (B) Western blot analysis of 10⁷ BSF and PCF WT cell equivalents. *TbPLA₁* was detected using antibodies raised against the recombinant protein. *, non-related background band to show loading control.

where *V_e* is the peak elution volume, *V_o* is the void volume and *V_t* is the total bed volume.

Purified His₆-tagged recombinant *TbPLA₁* (125 µl at 10 mg/ml dialysed against buffer) and a 13.7 kDa RNase A internal standard (125 µl at 3 mg/ml dissolved in buffer) were combined and loaded onto the gel-filtration column. The peak elution volumes and times were used to calculate their *K_{av}* values, and the molecular mass of the recombinant *TbPLA₁* was determined from the calibration curve. After the last sample had eluted, the column was immediately re-equilibrated in a buffer containing Triton X-100 (50 mM Tris/HCl, pH 7.5, 50 mM NaCl and 0.05 % Triton X-100), then a new calibration run was performed and another sample containing recombinant *TbPLA₁* spiked with RNase A was loaded and analysed as described.

RESULTS AND DISCUSSION

Life cycle stage expression of *TbPLA₁*

To initially examine the potential of PCF and BSF cells to synthesize lysoGPCho, an analysis of the expression of *TbPLA₁* in both these stages of the parasite life cycle was measured by Northern blotting. *TbPLA₁* mRNA levels are nearly equal in PCF and BSF cells (Figure 1A). However, Western blot analysis of

Figure 2 Validation of the generation of *TbPLA₁* mutants

(A) *TbPLA₁* null mutants were generated in BSF *T. brucei* after sequential *TbPLA₁* UTR targeted homologous recombination with UTR-flanked *puro^R* and *hyg^R* genes. Cell lines were analysed by Southern blotting after digestion of the genomic DNA with *AlwI*, which cuts *TbPLA₁* internally. *AlwI*-digested *T. brucei* WT DNA therefore reveals two fragments detectable by a

these cells offered a different perspective, since *TbPLA₁* levels in BSF cells were 3.6-fold greater than in PCF cells (Figure 1B). These findings justified an examination of the *in vivo* activity of *TbPLA₁* in BSF cells so that lysoGPCho synthesis in PCF and BSF cells could be compared.

Construction and integrity of *TbPLA₁* mutants

Before further comparative biochemical and enzymic studies could be performed, experiments, which led to the generation of a BSF double knockout of *TbPLA₁*, were warranted. Deletion constructs (5'-*puro^R*-3', 5'-*hyg^R*-3') targeted to the 5' and 3' flanking regions of the *TbPLA₁* ORF (open reading frame) were generated and the genes for *puro^R* and *hyg^R* were cloned between these flanking regions. These constructs were used to stably transform BSF parental cells that are referred to here as WT cells, these cells are commonly used in reverse genetic studies in *T. brucei*, and are transgenic clones that constitutively express T7 RNA polymerase and the TetR protein [26]. *TbPLA₁* is a single copy gene that does not appear to be essential for PCF cell viability in culture [4]. This seems also to be the case for BSF cells: two successive and successful rounds of gene replacement by UTR-targeted homologous recombination using the 5'-*puro^R*-3' and 5'-*hyg^R*-3' deletion constructs were sufficient to replace the two alleles of *TbPLA₁*, with no apparent resulting phenotype. Evidence for the generation of three BSF *TbPLA₁* null mutant cell lines ($\Delta pla1$) is shown by Southern blot analysis (Figure 2A).

To create an additional analytical tool, a genetic transformation was also carried out that attempted to generate a cell line ($\Delta pla1$ -rescPLA1-HA) that overexpressed a rescue copy of *TbPLA₁* in a $\Delta pla1$ background. To achieve this, a transformation was carried out using the *T. brucei* tetracycline-inducible overexpression vector pLEW82 [31], engineered to contain a recombinant form of *TbPLA₁* (Figure 2A). Upon tetracycline induction, trypanosomes transformed with pLEW82-PLA1-HA produce an ectopic copy of *TbPLA₁* with a C-terminal HA (haemagglutinin) tag (PLA1-HA). The resultant tetracycline-induced $\Delta pla1$ -rescPLA1-HA rescue clones produced *PLA1-HA* mRNA transcripts > 6-fold over the level of *TbPLA₁* mRNA in both the WT and their respective uninduced counterpart cells (Figure 2B).

The *TbPLA₁* rescue cells were examined for their ability to translate the overexpressed *TbPLA₁* mRNA. A Western blot probed with anti-*TbPLA₁* antibody revealed that expression of PLA1-HA was observed to be much less abundant than *TbPLA₁* mRNA in WT cells despite the high level of *PLA1-HA* mRNA overexpression (Figure 2C). It has been observed previously that lysoGPCho synthesis in *T. brucei* is regulated partly at the level of translation of *TbPLA₁* mRNA to control enzyme levels [4], therefore an up-regulation of *TbPLA₁* mRNA processing as a response to its overexpression in BSF $\Delta pla1$ -rescPLA1-HA mutant cells

fluorescein-labelled *TbPLA₁* ORF probe. In the three *TbPLA₁* null mutant cell lines ($\Delta pla1$) both *TbPLA₁* alleles are absent. A tetracycline (Tet) inducible HA-tagged recombinant ectopic copy of *TbPLA₁* (PLA1-HA) in pLEW82/PLA1-HA was introduced into a different locus in the $\Delta pla1$ cell line to produce a rescue cell line ($\Delta pla1$ -rescPLA1-HA). (B) Northern blot analysis of *TbPLA₁* mutants. Total RNA extracted from BSF WT and *TbPLA₁* mutant trypanosomes was resolved by electrophoresis, transferred on to nitrocellulose and hybridized with the ORF of *TbPLA₁* (top panel). β -Tubulin hybridization was used as a loading control (bottom panel). (C) Western blot analysis of *TbPLA₁* mutants (10^7 cell equivalents/lane). Both native PLA₁ (theoretically 32.4 kDa) and tagged PLA1-HA (theoretically 33.8 kDa) protein were separated by SDS/PAGE (15% gel) and detected by antibodies raised against the purified recombinant enzyme. (D) *In vitro* growth analysis of BSF *TbPLA₁* mutants. The logarithmic cumulative growth of the WT, null mutant and rescue cell lines is plotted as a function of culture duration. The population growth was calculated as cell density multiplied by the cumulative dilution factors obtained from daily passaging of the cells. *, non-related background bands that show loading in the null mutant lanes.

may account for a decrease in TbPLA₁ expression. The existence of regulatory-like processes to control TbPLA₁ concentration could be significant in the cell, considering the potential harm a membrane-degrading enzyme, such as TbPLA₁, could foster if expressed at uncontrollable levels.

The growth rate of cultured BSF TbPLA₁ mutants was assessed 2 months after the creation of the Δpla1-rescPLA1-HA rescue cell line. TbPLA₁ mutants are not compromised in their ability to divide and establish long-term cultures, and the population growth rate between the WT and TbPLA₁ mutants (under drug pressure) was not altered significantly (Figure 2D). Although TbPLA₁ is not essential in culture, it remained to be investigated whether or not, under more physiologically relevant conditions, the loss of TbPLA₁ would not be detrimental to the proper functioning of the cell. Thus, the virulence of BSF Δpla1 cells was compared with BSF WT cells in their capacity to establish an infection in an animal model system. Analysing the daily blood parasitaemia in inoculated mice revealed that the virulence of Δpla1 trypanosomes was not noticeably different from the WT: high parasitaemias of approx. 1.6×10^9 trypanosomes/ml of blood, which was nearly equal to that of the WT control, were reached within 72 h with an initial inoculation of 5×10^5 trypanosomes.

LysoGPCho analysis of TbPLA₁ mutants

Phospholipids are vital components of membranes and the phospholipases that modify them are recognized as important factors in mediating membrane dynamics. In PCF trypanosomes, it was shown that TbPLA₁ is a major lipolytic enzyme used by *T. brucei* to metabolize GPCho [4]. To show this, an efficient qualitative and quantitative method was employed to separate and characterize the major *T. brucei* phospholipid classes and their molecular species from total lipid extracts using nano-ESI-MS/MS. The same method was used in the present study to analyse the choline-phospholipid content of BSF cellular lipids by scanning for the collision-induced specific fragment ion *m/z* 184 (Figure 3A). The [M + H]⁺ (mass plus hydrogen positive ion) GPCho molecular species composition of BSF trypomastigotes was examined and compared with that determined previously from PCF cells (compare Figure 3A with Figure 5A of [4]). The pool of GPCho in both the PCF and BSF WT lipid extracts of *T. brucei* is comprised of the same molecular species of diacyl, alkylacyl and alkenylacyl lipids. However, BSF *T. brucei* appear to contain more lipids in the series GPCho_{38:y}.

To examine lysoGPCho species, however, the dominating diacyl and alkylacyl/alkenylacyl GPCho species were excluded from a subsequent analysis by scanning for *m/z* 184 precursors in the mass range *m/z* 400–700. Peak analysis of the resultant short range spectra from BSF WT lipids revealed the following set of [M + H]⁺ lipid metabolites: lysoGPCho_{-/22:6}, lysoGPCho_{-/22:5}, lysoGPCho_{-/22:4}, lysoGPCho_{-/18:2}, lysoGPCho_{-/18:1}, lysoGPCho_{-/18:0}, lysoGPCho_{-/20:2}, lysoGPCho_{-/20:3} and lysoGPCho_{-/20:4}. The peaks representing these metabolites and their [M + H]⁺ value are shown (Figure 3B, panel 1).

Qualitative comparison of the BSF lysoGPCho spectrum peaks with those from PCF analysis indicated that their abundance was increased significantly in the BSF spectra. To further investigate this result the lysoGPCho series metabolites were quantified by comparison with non-natural lysoGPCho_{17:0/-} and lysoGPCho_{24:0/-} added to each sample prior to lipid extraction (Figure 3B, inset to panel 1). From these quantitative ESI-MS/MS experiments it was perceived that BSF WT cells possessed 3-fold the number of moles of total lysoGPCho than in PCF WT cells (Table 1). However, this lysoGPCho increase was not distributed evenly among the major lysoGPCho series. There was a 3.9-fold

increase in the lysoGPCho_{-/22:y} series whereas only a 1.3-fold increase was observed in the lysoGPCho_{-/20:y} series. It is, however, important to recall that an observed increase or decrease in a lysoGPCho metabolite doesn't necessarily equate to its increased or decreased rate of synthesis; the change could be accounted for by an increase or decrease in the rate at which these intermediates are metabolized further.

The synthesis of lysoGPCho molecules in BSF *T. brucei* are mediated by TbPLA₁ since Δpla1 cells show a very significant drop in their levels (Figure 3B, panel 2). The number of moles of lysoGPCho in BSF Δpla1 null mutants was recorded to be approx. 20 % of those metabolite levels in WT cells (Table 1 and Figure 3B, inset to panel 2). The individual lysoGPCho_{-/18:y}, lysoGPCho_{-/20:y} and lysoGPCho_{-/22:y} series levels decreased by 77 %, 60 % and 84 % in the BSF TbPLA₁ null mutants respectively. This decline in the ability to synthesize lysoGPCho was confirmed to be the direct result of the loss of TbPLA₁, since tetracycline-induced Δpla1-rescPLA1-HA cells rescued lysoGPCho synthesis (Figure 3B, panels 3 and 4). This recovery is only modest due to the small amount of PLA1-HA synthesized from overexpressed PLA1-HA mRNA (Figure 2C). The individual lysoGPCho_{-/18:y}, lysoGPCho_{-/20:y} and lysoGPCho_{-/22:y} series levels were restored to 41 %, 73 % and 46 % of normal BSF levels of these metabolites in these cells respectively (Table 1 and Figure 3B, panels 3 and 4 and their respective inset panels).

TbPLA₁ null mutants still possess a small amount of lysoGPCho. There are several explanations for this observation. It is possible that one or more alternative phospholipases is responsible for a low level of synthesis *in vivo*. However, lysates of PCF Δpla1 cells could no longer produce any lysoGPCho in an *in vitro* assay [4], and this is also true for BSF Δpla1 cells (results not shown). Much of the lysoGPCho peak area in Δpla1 cells could be contributed by background levels of fragment ions formed mechanically from diacyl-GPCho during the sample preparation and/or the ionization process. Also, BSF *T. brucei* is known to efficiently uptake exogenous lysoGPCho from both culture and the host's bloodstream [18,20]; therefore, some of the unsaturated lysoGPCho species present in Δpla1 mutants could be from low-density-lipoprotein- or albumin-linked unsaturated lysoGPCho from host plasma, the abundance of which are estimated to be 4.2 and 25.9 nmol/ml of human plasma respectively [23].

Kinetic analysis of lysoGPCho synthesis

TbPLA₁ is a soluble enzyme that, both *in vivo* and *in vitro*, acts on insoluble lamellar GPCho substrates. In order to achieve lysoGPCho synthesis TbPLA₁ therefore must: 1) adsorb to membrane surfaces, i.e. undergo interfacial activation, and 2) cleave the *sn*-1 ester of its substrate. The mechanism by which the latter is accomplished is through the action of a Ser-His-Asp catalytic triad [4]. The following experiments give insight into the mechanism of interfacial activation and binding properties of TbPLA₁.

Analytically, the actions of soluble lipolytic enzymes can be manifested by employing 'surface dilution' kinetics [28–30], and this can be applied to give insight into the mechanism by which an interfacial enzyme initially binds to an aggregate [32] (see the Experimental section for an explanation of this kinetic scheme). Surface dilution kinetics for recombinant TbPLA₁ were carried out with the use of mixed micelles composed of GPCho_{16:0/16:0}, Bis-BODIPY® FL-C₁₁-PC and Triton X-100. Surface dilution was accomplished by adding the non-ionic detergent Triton X-100 in increments while keeping the substrate concentration constant. TbPLA₁ activity at various substrate surface concentrations (0.036, 0.018 and 0.009 mole fractions) was then measured as a function of various substrate bulk concentrations.

Figure 3 TbPLA₁-mediated lysoGPCho synthesis in BSF *T. brucei*

(A) GPCho composition of *T. brucei* BSF WT cells spiked with the internal standards lysoGPCho_{-17:0} and lysoGPCho_{-24:0}. The spectrum was acquired from positive-ion ESI-MS/MS precursor ion scanning for m/z 184 ions. Identities of the major $[M + H]^+$ ions are indicated. (B) Short-range ESI-MS/MS spectra in positive-ion mode, showing the parents of the m/z 184 ion from total lipid extracts from WT and TbPLA₁ null cell lines. The lysoGPCho $[M + H]^+$ metabolites detected are boxed and annotated next to the m/z peak from which they derived. $x:y$ refers to the total number of *sn*-2 FA carbon atoms (x) and their degree of unsaturation (y). The inset panels show short-range spectra from lipid extracts from each cell line that were spiked with lysoGPCho internal standards.

First, an experiment was carried out to examine the possibility that the enzyme binds non-specifically to the micelle surface, known as the surface-binding model. In this instance the aggregate molecules are expressed as the molar concentration of both the lipid and detergent that make up the aggregate. Comparison of TbPLA₁ activity at various surface concentrations shows that as the surface concentration of lipid decreases there is a decrease in the apparent V_{max} , this is the surface dilution phenomenon (Figure 4A). The true V_{max} is the V_{max} at an infinite mole fraction of phospholipid substrate. The linear relationship between the

decrease in activity as the surface concentration of substrate decreases also shows that TbPLA₁ does not have a strong affinity for individual Triton X-100 molecules, since they are in great excess over the substrate at all mole fractions.

Theoretical predictions for the surface-binding model state that in a double-reciprocal plot for Figure 4(A) the lines should converge at a point where its y-axis ordinate represents $1/V_{max}$ (Figure 4B). The presented linear regression lines converge adequately, but kinetic constants were more easily obtained using the $1/v$ intercepts from Figure 4(B) in a replot against the

Table 1 Quantification of lysoGPCho in WT and BSF TbPLA₁ mutants

The lysoGPCho content of lipid extracts from 10^8 *T. brucei* cells was measured with lysoGPCho_{17:0/-} and lysoGPCho_{24:0/-} as internal standards. Values are from a typical analysis and are presented as pmol/ 10^8 cells. Tet, tetracycline.

Cell line	LysoGPCho series* (pmol/ 10^8 cells)				Total
	Tet	-/18:y	-/20:y	-/22:y	
WT (PCF)		53	31	72	156
WT (BSF)		143	40	280	463
Δ pla1		33	16	45	94
Δ pla1-rescPLA1-HA	—	35	20	48	103
Δ pla1-rescPLA1-HA	+	59	29	128	216

* Integration of m/z $[M + H]^+$ signals to obtain peak areas was performed by assembling individual lysoGPCho into a 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 various degrees of unsaturation, represented by 'y'.

reciprocal of the mole fractions from which they were derived (Figure 4C). The data from this plot produce a straight line where the intercept of the $1/v$ axis represents the true $1/V_{\max}$, and the $1/[\text{mole fraction}]$ intercept is the $-1/K_m$, where K_m is the interfacial Michaelis constant for the mixed micelle. The $V_{\max}$ and K_m towards GPCho_{16:0/16:0} were calculated to be $487 \mu\text{mol}/\text{min}$ per mg and 0.22 respectively. The surface dilution kinetics

equations also predict that if the kinetic model being employed is correct, then a replot of the slopes of the lines in Figure 4(B) versus the reciprocal of the mole fractions should be linear and pass through the origin, as seen in our replot (Figure 4D). The data presented seem to fit the surface-binding model.

It is also feasible that the enzyme could bind specifically to a lipid substrate in the micelle with one binding domain, forming EA, prior to specifically binding to another lipid molecule for catalysis; this case has been termed the dual phospholipid-binding model [29,30]. Analysis can be carried out according to this model using the same data used to test the first model [33]. Accordingly, a plot of PLA₁ activity as a function of the molar concentration of GPCho_{16:0/16:0} shows again that the enzyme activity is dependent on surface concentration of substrate (Figure 5A). The double reciprocal plot of the data in Figure 5(A) shows that the linear regression lines of the data do not converge as predicted in the dual phospholipid-binding model (Figure 5B). A replot of the $1/v$ intercepts from the three data sets used in Figure 5(B) versus the reciprocal of the mole fraction produces the same result as in Figure 5(C) and the K_m and $V_{\max}$ values are thus identical. A plot of the slopes in Figure 5(B) versus the reciprocal of the mole fraction produces a straight line that does not pass through the origin, which suggests that the data do not fit the dual phospholipid-binding model.

Kinetic analysis thus appears to indicate that TbPLA₁ activation *in vitro* is mediated by non-specific binding to the mixed micelle

Figure 4 TbPLA₁ activity in relation to the surface-binding model

(A) The fluorescent BODIPY[®] C₁₁-PC assay was used to determine the rate of PLA₁ velocity (V) as a function of varying bulk concentrations of GPCho_{16:0/16:0} plus Triton X-100 at set mole fractions (X_s). The results are the average for three separate experiments each performed in triplicate. The data points varied by less than one S.D., and the overlapping ranges have been omitted for clarity. (B) Reciprocal plot of the data (A). (C) Replot of the $1/V$ intercepts from (B) versus the reciprocal of the mole fraction. (D) Replot using the slopes obtained in (B) versus the reciprocal of the mole fraction from which they were derived. The lines drawn in (B–D) were fitted to the data after linear regression analysis.

Figure 5 TbPLA₁ activity in relation to the dual phospholipid-binding model

(A) The fluorescent BODIPY[®]-C₁₁-PC assay was used to determine the rate of PLA₁ velocity (V) as a function of varying only bulk concentrations of GPCho_{16:0/16:0} at set mole fractions (X_s). The results are the average for three separate experiments each performed in triplicate. The data points varied by less than one S.D., and the overlapping ranges have been omitted for clarity. (B–D) are the same as described in the legend for Figure 4 (B–D).

interface, prior to binding and catalysing a phospholipid substrate. The use of Triton X-100–GPCho_{16:0/16:0}–Bis-BODIPY[®] FL-C₁₁-PC mixed micelles in a 96-well plate assay to perform kinetics greatly facilitated the experimental procedure and improved upon some drawbacks experienced in previously used assay methods, which were more laborious, lacked sensitivity [28] and/or used unnatural thiol ester substrate analogues, which are poor substrates compared with oxyester lipids [29,34].

Evidence for EA formation

The kinetic studies provided fundamental insight into the mechanism of activation of recombinant TbPLA₁, but making a conclusion that interfacial activation is substrate independent could not be done until it had been verified independently. If the first kinetic model is accurate, TbPLA₁ should be able to bind to an aggregate whose surface is devoid of phospholipid substrate and composed solely of non-ionic diluters. To test this prediction, a Superdex 200 size-exclusion column equilibrated in the absence or presence of lipid-free Triton X-100 micelles was loaded with a solution containing purified recombinant TbPLA₁. The sample was spiked with RNase A as an internal standard and the elution absorbance was monitored at UV_{280 nm}. In the absence of an interface, the majority of TbPLA₁ was shown by peak area analysis to elute in monomer form (38.7 kDa; 96%), whereas a small proportion of enzyme eluted in the void volume and as a 'dimer' (75.9 kDa) (Figure 6).

In contrast, the affinity of recombinant TbPLA₁ for a pure Triton X-100 micelle surface is demonstrated by a shift in its elution time from 29.7 to 25.8 min, representing an increase in apparent molecular mass from 38.7 kDa to 134 kDa (Figure 6). This increase of 95.3 kDa corresponds very well to an increase in molecular mass if a monomer of the enzyme were bound to exactly one micelle of Triton X-100, which has a molecular mass of approx. 90 kDa [35].

These results provide evidence for enzyme–aggregate formation and are a testament to the non-specific adsorption mechanism employed by this enzyme during interfacial activation onto mixed micelles. This association to the micelle surface is not dependent on surface charge, since Triton X-100 is non-ionic, and it is also not due to specific binding to Triton X-100 molecules (Figure 4A). The non-specific nature of binding to Triton X-100 mixed micelles for TbPLA₁ contrasts with the nature of binding for cytosolic PA–PLA₁ partially purified from bovine testis, which requires anionic phosphoglycerides to bind to surfaces [36]. No other PLA₁s have been examined for their binding properties.

Metal ion effects on lysoGPCho formation

To understand more precisely the fundamental basis behind lysoGPCho formation by TbPLA₁, its biochemical properties and activity were examined further *in vitro*. Besides a requisite of an interface for activation, some phospholipases require co-factors for optimal activity, whereas others do not. For example, cytosolic PLA₂ activity is calcium-dependent, whereas another PLA₂

Figure 6 Adsorption of TbPLA₁ on to detergent micelles

Size-exclusion chromatography elution profiles of a sample of recombinant TbPLA₁ (1.25 mg) spiked with an RNase A internal standard (13.7 kDa) are plotted as a function of their UV absorbance at 280 nm (A_{280}). The peak elution times of TbPLA₁ in the absence (29.7 min) or presence (25.8 min) of Triton X-100 micelles were compared with the molecular mass standard elution times (shown above the top x-axis) in order to calculate an approximate molecular mass shift (see the Experimental section for details). The RNase A internal standard (35.9 min) and void volume (16 min) peaks displayed equivalent elution times in both buffers. Peak identities were confirmed by SDS/PAGE of the eluate fractions (results not shown).

Table 2 Phospholipase A₁ activity with various compounds

All compounds were pre-incubated with enzyme (400 pg) for 10 min at the listed concentrations before commencing the reaction by the addition of substrate to a final concentration of 0.075 mM. Activity was measured using the fluorescent BODIPY® C₁₁-PC assay at pH 7.0 for 32 min, with a mole fraction of 0.018. Values are presented as the percentage of activity observed relative to the 'enzyme only' control. Results are shown as averages $\pm$ S.D. for two experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.005$.

Additive	% Relative activity			
	2 μ M	20 μ M	200 μ M	2 mM
None†	100	100	100	100
MgCl ₂	98 $\pm$ 3	85 $\pm$ 9	94 $\pm$ 3	96 $\pm$ 2
CaCl ₂	101 $\pm$ 2	91 $\pm$ 5	92 $\pm$ 10	87 $\pm$ 9
MnCl ₂	97 $\pm$ 5	99 $\pm$ 2	104 $\pm$ 2	87 $\pm$ 6
FeCl ₂	83 $\pm$ 4	76 $\pm$ 7	20 $\pm$ 2*	0 $\pm$ 0**
CoCl ₂	99 $\pm$ 0	101 $\pm$ 0	90 $\pm$ 5	57 $\pm$ 2*
NiCl ₂	102 $\pm$ 1	94 $\pm$ 3	85 $\pm$ 7	43 $\pm$ 10*
CuCl ₂	55 $\pm$ 4*	6 $\pm$ 1**	0 $\pm$ 0**	0 $\pm$ 0**
ZnSO ₄	105 $\pm$ 1	93 $\pm$ 4	59 $\pm$ 9*	34 $\pm$ 6*
CsCl ₂	101 $\pm$ 2	112 $\pm$ 1	112 $\pm$ 0	102 $\pm$ 2
CdCl ₂	81 $\pm$ 5	37 $\pm$ 8*	1 $\pm$ 1**	0 $\pm$ 0**
EDTA‡	98 $\pm$ 0	94 $\pm$ 4	91 $\pm$ 5	96 $\pm$ 2

† Enzyme only control, set at 100% (26 μ mol/min per mg).

‡ The maximum concentration of EDTA added was 10 mM, resulting in 91% relative activity.

group, iPLA₂, is calcium-independent [37]. Conversely, the heavier metals can be potent inhibitors of lipase activity [17,38]. In the present study, both the metal ion co-factor and inhibitory profile for TbPLA₁ was assessed.

TbPLA₁ apparently has no absolute metal ion requirements for activity, since dialysis or incubation of the enzyme with EDTA prior to commencing the reaction did not abolish lysoGPCCho synthesis (Table 2). This result is consistent with a previous

Table 3 TbPLA₁ activity in the presence of various inhibitors

All inhibitors were pre-incubated with enzyme (600 pg) for 10 min at the listed concentrations and conditions before commencing the reaction by the addition of a substrate to a final concentration of 0.075 mM. Activity was measured using the fluorescent BODIPY® C₁₁-PC assay at pH 7.0 for 32 min, with a mole fraction of 0.018. Values are presented as the percentage of activity observed relative to the 'enzyme only' control. Results are shown as averages $\pm$ S.D. for three experiments performed in triplicate. *, $P < 0.05$; E-600, diethyl-*p*-nitrophenyl phosphate; PACOCF₃, palmitoyl trifluoromethyl ketone analogue; AACOCF₃, arachidonyl trifluoromethyl ketone analogue.

Serine modifier	% Relative activity		
	10 mM	1 mM	0.1 mM
None†	100	100	100
iPr ₂ P-F	79 $\pm$ 9*	104 $\pm$ 1	99 $\pm$ 0
PMSF	—	91 $\pm$ 6	95 $\pm$ 3
E-600	72 $\pm$ 8*	89 $\pm$ 7	99 $\pm$ 3
AACOCF ₃	87 $\pm$ 4*	99 $\pm$ 0	100 $\pm$ 1
PACOCF ₃	61 $\pm$ 4*	98 $\pm$ 1	98 $\pm$ 3

† Enzyme only control set at 100% (38 μ mol/min per mg).

finding that PLA₁ activity in lysates of *T. brucei* is divalent cation independent [39]. Also, magnesium and manganese did not enhance or alter TbPLA₁ activity, as has been observed previously for mammalian PLA₁ [40]. On the other hand, a number of heavy metals inhibited TbPLA₁ activity at various concentrations, a phenomenon that has been observed before for PLA₁ activity in soluble fractions of *T. brucei* incubated with 5 mM metal ions [41]. The most potent metals are cadmium and copper, whose inhibitory effects can be observed at concentrations as low as 2 μ M (Table 2). Incubation with different levels of iron produced partial to total inhibition, whereas moderate inhibition was detected in the presence of relatively high concentrations of nickel and zinc. The inhibitory effects to TbPLA₁ *in vitro* upon pre-incubation with heavy metals are mediated by an undefined mechanism.

Effects of serine inhibitors on TbPLA₁ activity

Knowing that Ser¹³¹ is the active-site residue for TbPLA₁ [4], the next line of experiments sought to inhibit enzyme activity with the known active-site serine modifiers iPr₂P-F (di-isopropyl fluorophosphate), PMSF and E-600 (diethyl-*p*-nitrophenyl phosphate). It was anticipated that iPr₂P-F would have little effect on PLA₁ activity originating from *T. brucei* [41]. Palmitoyl and arachidonyl trifluoromethyl ketone analogues (PACOCF₃ and AACOCF₃ respectively) were also examined for potential effects of product inhibition, as observed for cytosolic PLA₂ enzymes when in the presence of these compounds [42]. However, pre-incubation with moderate levels of all of these individual compounds with TbPLA₁ resulted in no inhibition, and only relatively little inhibition of PLA₁ activity was observed with a very high concentration of inhibitor (Table 3). These inhibitors covalently and permanently modify accessible serine residues, and, upon successful modification, a complete loss of activity would be anticipated. The fact that TbPLA₁ is insensitive to working concentrations of these serine-modifying reagents suggests that the serine active-site residue of TbPLA₁ is inaccessible. Many prokaryotic and eukaryotic lipases, which structurally have single and multiple domains respectively, contain active-sites buried within the enzyme that are sheltered by an α -helical lid structure, rendering the catalytic triad un-solvated [43,44]. The active-site of a lipase is able to cleave the substrate only after interfacial activation, and the α -helical lid structure plays a role in this enzyme activation [45]. In light of the failure of serine-modifying

reagents to inhibit TbPLA₁, these results suggest that the active-site of TbPLA₁ may also be covered by a similar lid structure when the enzyme is not adsorbed to membrane surfaces, and that interfacial activation of TbPLA₁ engenders a conformational change to expose these otherwise inaccessible active-site elements that are needed for phospholipid hydrolysis, in a structural change that may be similar to lipase activation. Structural studies are underway to clarify this issue.

Conclusions

TbPLA₁ is constitutively expressed in both PCF and BSF trypanosomes. A reverse genetics approach was employed to study the effects of TbPLA₁ deletion in BSF parasites. Ablation of TbPLA₁ resulted in a very drastic reduction of all the molecular species of lysoGPCho metabolites that were present in WT cells. Interestingly, despite the adoption and adaptation of TbPLA₁ in the PCF life cycle stage [4], lysoGPCho levels in the BSF stage are 3-fold greater than in the PCF stage. TbPLA₁ does not appear to be essential for cell viability in culture or for virulence in a mammalian host. Evidence that the enzyme is insensitive to serine-modifying reagents suggests that the catalytic triad active-site of TbPLA₁ is buried inside the enzyme and is sheltered by a lid domain, a property shared with other lipases. Furthermore, in a GPCho–Triton X-100 mixed micelle system, this soluble, monomeric enzyme appears to adsorb to lipid–micelle interfaces non-specifically, instead of requiring lipid substrates to achieve interfacial activation. On balance, the results suggest that this binding and activation mechanism could help to induce a favourable conformational change, which is thought to be needed to expose active-site elements.

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