Phosphatidylserine synthase 2 and phosphatidylserine decarboxylase are essential for aminophospholipid synthesis in *Trypanosoma brucei*

Luce Farine,1 Jennifer Jelk,1 Jae-Yeon Choi,2 Dennis R. Voelker,2 Jon Nunes,3 Terry K. Smith3* and Peter Butikofer1*

1 Institute of Biochemistry and Molecular Medicine, University of Bern, Bern 3012, Switzerland.
2 Department of Medicine, National Jewish Health, Denver, CO 80206, USA.
3 Biomedical Sciences Research Complex, University of St. Andrews, St. Andrews, Scotland.

Summary

Phosphatidylethanolamine (PE) and phosphatidylserine (PS) are ubiquitously expressed and metabolically interconnected glycerophospholipids in eukaryotes and prokaryotes. In *Trypanosoma brucei*, PE synthesis has been shown to occur mainly via the Kennedy pathway, one of the three routes leading to PE synthesis in eukaryotes, while PS synthesis has not been studied experimentally. We now reveal the importance of *T. brucei* PS synthase 2 (TbPSS2) and *T. brucei* PS decarboxylase (TbPSD), two key enzymes involved in aminophospholipid synthesis, for trypanosome viability. By using tetracycline-inducible down-regulation of gene expression and *in vivo* and *in vitro* metabolic labeling, we found that TbPSS2 (i) is necessary for normal growth of procyclic trypanosomes, (ii) localizes to the endoplasmic reticulum and (iii) represents the unique route for PS formation in *T. brucei*. In addition, we identified TbPSD as type I PS decarboxylase in the mitochondrion and found that it is processed proteolytically at a WGSS cleavage site into a heterodimer. Down-regulation of TbPSD expression affected mitochondrial integrity in both procyclic and bloodstream form trypanosomes, decreased ATP production via oxidative phosphorylation in procyclic form and affected parasite growth.

Introduction

Glycerophospholipids are major building blocks of all biological membranes. The relative abundance of the different classes and their subclass and molecular species compositions not only modulate membrane characteristics, such as fluidity, curvature and membrane tension, but also affect properties of membrane-associated proteins (reviewed by Lee, 2004). The aminophospholipid classes, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are present in membranes of most eukaryotes and prokaryotes (reviewed by Vance, 2008; Vance and Tasseva, 2013). PE has been shown to be involved in a wide range of biological processes, including cell division (Emoto et al., 1996) and protein folding (Bogdanov and Dowhan, 1998), and represents the ethanolamine donor for the synthesis of glycosylphosphatidylinositol (GPI) anchors (Menon and Stevens, 1992) and other protein modifications (Ichimura et al., 2000; Signorell et al., 2008a; Cullen and Trent, 2010). PS functions as an important precursor for some pools of PE (reviewed by Vance and Steenberg, 2005), acts as a cofactor for enzymes involved in signaling pathways (Takai et al., 1979), and its exposure at the cell surface is an early event in apoptosis (Fadok et al., 1992) and serves as a critical cofactor in blood clotting (Bevers et al., 1983). The pathways for the synthesis of PS and PE are often coupled, but the contributions of the individual pathways and their interconnections differ considerably among eukaryotes (reviewed by Vance and Tasseva, 2013).

Decarboxylation of PS represents a main route for PE synthesis in many organisms (Borkenhagen et al., 1961; reviewed by Schuiki and Daum, 2009). It is the major pathway for PE production in bacteria and the major route in *Saccharomyces cerevisiae*, however it is also active in mammals and plants. In addition, in mammalian cells, plants and yeast, PE can be formed via the
CDP-ethanolamine branch of the Kennedy pathway (Kennedy and Weiss, 1956; reviewed by Vance, 2008; Gibellini and Smith, 2010). A third pathway for PE synthesis in many eukaryotes involves head group exchange with PS (Suzuki and Kanfer, 1985; reviewed by Vance, 2008). PS decarboxylases (PSDs) in prokar-
yotes and eukaryotes consist of two different types. Type I PSDs are present in eukaryotic mitochondria and bacteria whereas type II PSDs comprise eukaryotic enzymes located in the endomembrane system (Golgi, endoplasmic reticulum (ER), vacuole/tonoplast) (reviewed by Schuiki and Daum, 2009). PSDs are usually transmembrane proteins that are active as heterodimers composed of an α- and a β-subunit. The subunits are generated from a proenzyme via autocata-
lytic cleavage at a conserved recognition motif close to the C-terminus (GSS/T), generating a long N-terminal (transmembrane) β-subunit and a short C-terminal α-
subunit (Trotter et al., 1995; Kitamura et al., 2002). PSDs contain an unusual pyruvolyl prosthetic group, which is generated at the amino terminus of the α-
subunit from the serine residue located at the cleavage site of the proenzyme. Although the two PSD subunits remain tightly associated after the cleavage, they are not covalently linked (reviewed by van Poelje and Snell, 1990; Schuiki and Daum, 2009).

Bacteria and mammalian cells contain a single type I PSD, while S. cerevisiae express a mitochondrial type I PSD (responsible for 80% of PSD activity) and a type II PSD localized in the Golgi (reviewed by Vance and Steenbergen, 2005; Schuiki and Daum, 2009). Yeast mutants lacking both PSD enzymes are auxotrophic for ethanolamine, which is used for PE synthesis via the CDP-ethanolamine pathway (Trotter and Voelker, 1995). Plants contain a mitochondrial psd1 and two type II PSDs localized in the endomembrane system (Nerlich et al., 2007). Interestingly, unique forms of PSD have been identified in protozoan parasites. An ER-localized type I PSD has been described in Plasmodium falciparum (Baunaure et al., 2003), representing the only known non-
mitochondrial type I PSD so far, whereas a soluble PSD secreted into the parasitophorous vacuole has been described in Toxoplasma gondii (Gupta et al., 2012).

PS can be synthesized by two different pathways. PS synthase (PSS) uses CDP-diacylglycerol (CDP-DAG) and serine as substrates, while PS synthase 1 (PSS1) and PS synthase 2 (PSS2) generate PS via calcium-
dependent head group exchange reactions using PC and PE, respectively, as substrates. PSS enzymes are membrane-bound and have been detected in membrane fractions of bacteria (Matsumoto, 1997) and in the outer mitochondrial and ribosomal fraction of yeast cells (Yamashita and Nikawa, 1997). Depletion of PSS causes a conditional lethal temperature-sensitive growth phenotype in Escherichia coli (Ohta and Shibuya, 1977) and renders yeast auxotrophic for ethanolamine/choline (Nikawa and Yamashita, 1981). In contrast, mammalian PSS1/2 enzymes have been localized to mitochondria-
associated membranes (MAMs) (Stone and Vance, 2000). MAMs represent a particular membrane fraction with distinct biochemical properties harboring proteins that are involved in the synthesis and transport of lipids and mediate import of PS into mitochondria for decar-
boxylation to PE (reviewed by Vance, 2014). Comparative schemes with biosynthetic connections between PC, PE, and PS in eukaryotes are shown in Supporting Information Fig. S1.

The pathways for de novo synthesis of PE have also been studied in the very early-branching eukaryote Tryp-
anosoma brucei (reviewed by Serrichio and Bütkofer, 2011; Farine and Bütkofer, 2013). T. brucei is the caus-
ative agent of human African trypanosomiasis, also called sleeping sickness, and nagana a related livestock disease. The diseases are fatal unless treated and repre-
sent a major cause of poverty in sub-Saharan Africa (Fevre et al., 2008). Trypanosomes alternate between an insect vector, the tsetse, where they replicate as procyclic forms in the midgut and as epimastigotes in the salivary glands, and a mammalian host, where they replicate as long slender bloodstream forms (Vickerman, 1985).

It has been shown previously that the CDP-
ethanolamine branch of the Kennedy pathway is essential for growth of T. brucei procyclic and bloodstream forms in culture (Gibellini et al., 2008; Signorell et al., 2008b; Gibellini et al., 2009; Signorell et al., 2009). In addition, experimental data in procyclic forms (Signorell et al., 2008b) together with bioinformatic analysis of the T. bru-
cei genome suggested that two other possible routes may contribute to PE formation in T. brucei: decarboxyla-
tion of PS by putative TbPSSD (Tb927.9.10080) and head group exchange with PS by putative TbPSSS2 (Tb927.7.3760). However, the respective enzymes have not been characterized experimentally and their essentiality has not been investigated. In the present study, we characterize TbPSSD as mitochondrial type I PSD. Its down-regulation affects cell growth, mitochondrial integrity and ATP produc-
tion by oxidative phosphorylation. In addition, we demon-
strate that base exchange between PE and PS is catalyzed by TbPSSS2 and represents the only pathway for PS produc-
tion in T. brucei. Expression of TbPSSS2 is essential for nor-
mal growth of T. brucei in culture.

**Results**

*Tb927.7.3760 encodes a PS synthase 2*

*In silico* analysis of putative TbPSSS2 (Tb927.7.3760) protein sequence revealed the presence of 8 to 9
membrane spanning regions (Phobius, TriTrypDB) and a domain between amino acids 135 to 412 belonging to the PSS pfam family (PF03034). The PSS family comprises base exchange enzymes that replace the existing head group of a phospholipid with L-serine, such as mammalian PSS1 and PSS2 (Finn et al., 2014). PSS enzymes are mechanistically not related to PS synthases from yeast (CHO1), which are members of the CDP-alcohol phosphatidylinositoltransferase protein family. On the amino acid level, bioinformatics prediction tools (Pairwise Sequence Alignment, EMBOSS, EMBL-EBI) revealed 23.7% and 26.2% identity (37.1% and 39.4% similarity) between TbPSS2 and human PSS1 (isoform 1) and PSS2, respectively, but only 11.8% identity (19.8% similarity) between TbPSS2 and S. cerevisiae PSS, which is consistent with TbPSS2 representing a base exchange enzyme. This was confirmed by over-expressing TbPSS2 in E. coli (Supporting Information Fig. S2A) and assaying a membrane preparation for PSS activity in the presence of various substrates (Supporting Information Fig. S2B). Low PSS activity was observed in the presence of CDP-DAG as substrate, likely due to the action of endogenous E. coli PSS. In contrast, in the presence of PE, strong head group exchange activity with [3H]serine was observed in TbPSS2-overexpressing membranes compared to control membranes.

**Down-regulation of TbPSS2 inhibits growth of procyclic form trypanosomes**

Essentiality of TbPSS2 for parasite growth was assessed using tetracycline-inducible RNAi-mediated down-regulation of TbPSS2. After 3 days of tetracycline induction, TbPSS2 mRNA levels showed efficient down-regulation (Fig. 1A) and parasite growth in the presence of tetracycline was reduced compared to uninduced cells after 6 days of culture, demonstrating that
expression of TbPSS2 is essential for normal growth of T. brucei procyclic forms in culture.

**TbPSS2 co-localizes with the ER marker BiP**

Localization of TbPSS2 in T. brucei procyclic forms was studied by expressing an inducible N-terminally 3xc-myc-tagged form of TbPSS2. Immunofluorescence microscopy revealed good co-localization of 3xc-myc-TbPSS2 with the ER marker BiP, whereas no co-localization was observed with mitochondrial mtHSP70 (Fig. 1B).

**TbPSS2 is the only route for PS synthesis in T. brucei**

To study the importance of TbPSS2 on PS synthesis in procyclic forms, RNAi-suppressed parasites were labeled with [3H]serine and lipids were analyzed by one- and two-dimensional thin layer chromatography (TLC) in combination with radioisotope scanning, fluorography and lipid phosphorus determination. Incubation of trypanosomes with [3H]serine is expected to not only label PS and PE (via decarboxylation of [3H]PS), but also the major sphingolipids, inositol phosphorylceramide (IPC) and sphingomyelin (SM), via serine palmitoyltransferase (Signorell et al., 2008b). Since degradation of [3H]serine-labeled sphingolipids via sphingosine-1-phosphate lyase may result in the formation of [3H]ethanolamine-phosphate, which is prevalent in Leishmania (Zhang et al., 2007), which in turn can be used for [3H]PE synthesis via the Kennedy pathway, an inhibitor of serine palmitoyltransferase, myriocin, was used to block de novo synthesis of sphingolipids. As shown in Fig. 2A, and consistent with the Leishmania results (Zhang et al., 2007), the addition of myriocin completely blocked de novo formation of IPC and SM in T. brucei procyclic forms in culture, resulting in labeling of PS and PE only. These conditions were subsequently used to study [3H]serine-labeling in parasites after down-regulation of TbPSS2. The results show that RNAi against TbPSS2 for 3 days inhibited incorporation of [3H]serine into newly synthesized PS by 94.8 ± 1.0% (mean value ± standard deviation from three independent

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**Fig. 2.** Lipid analysis of TbPSS2 parasites after [3H]serine labeling. Procyclic form trypanosomes or digitonin extracts were labeled with [3H]serine for 4 h and lipids were extracted, separated by one-dimensional thin layer chromatography and detected using a radioisotope detector.

A. [3H]Serine labeling of phospholipids in parasites before (left panel) and after 30 min of myriocin treatment (2.5 μM; right panel).
B, C. [3H]Serine labeling of whole cells (panel B) and digitonin extracts (panel C) before and after RNAi-mediated depletion of TbPSS2 for 6 days.
D. [3H]Serine labeling of control and 3xc-myc-TbPSS2 overexpressing procyclic forms after 2 days of tetracycline induction. The scans are representative of at least three independent experiments.
experiments) in intact parasites (Fig. 2B) and by 91.3 ± 1.0% (mean value ± standard deviation from three independent experiments) in digitonin extracts from TbPSS2-depleted parasites (Fig. 2C). Interestingly, [3H]PE formation was not affected by down-regulation of TbPSS2 (Fig. 2B) (remaining at 92.5 ± 14.6% of control levels; mean value ± standard deviation from three independent experiments) and no labeling of PE was observed in digitonin extracts from control and TbPSS2-depleted cells (Fig. 2C). In addition, quantification by two-dimensional TLC and lipid phosphorus determination of total phospholipids after down-regulation of TbPSS2 for 4 days revealed a reduction in the steady-state levels of PS from 3.0 ± 1.1% of total phospholipids in control parasites to 0.9 ± 0.2% in TbPSS2-depleted cells (mean values ± standard deviations from four independent experiments), indicating decreased activity of TbPSS2 resulting in lower PS levels. This finding is consistent with the observation that PS could no longer be labeled with [3H]serine in vivo in trypanosomes after down-regulation of TbPSS2 (Supporting Information Fig. S3). Together, these results identify TbPSS2 as the only route for de novo PS synthesis in T. brucei.

In addition, to study whether the N-terminally tagged TbPSS2 used for sub-cellular localization (see Fig. 1B) is functional, digitonin extracts from parasites expressing (inducible) 3xc-myc-TbPSS2 were assayed for enzyme activity. The results show increased (i.e. 2.2 ± 0.3-fold; mean value ± standard deviation from three independent experiments) [3H]PS formation in extracts from TbPSS2 over-expressing parasites compared to extracts from the same cell line before induction of 3xc-myc-TbPSS2 expression (Fig. 2D), indicating that tagged TbPSS2 is catalytically active.

Tb927.9.10080 encodes a type I PS decarboxylase

Using prediction programs and amino acid sequence comparison tools, we identified Tb927.9.10080 as a candidate gene encoding putative T. brucei type I PSD (TbPSD). The sequence revealed a predicted mitochondrial targeting sequence (I-Psiort prediction; Bannai et al., 2002) and a single transmembrane domain in the N-terminal part of the protein (SMART; Schultz et al., 1998). The pfam domain PS Decarboxylase (PF02666) was located between amino acids 125 and 348 (Finn et al., 2014). Amino acid sequence alignments of TbPSD with E. coli, P. falciparum, T. gondii, Arabidopsis thaliana and human PSDs showed between 19.2 and 24.6% identity and revealed the presence of a putative proteolytic PSD cleavage recognition motif, WGSS (amino acids 316-319) (Fig. 3A). The same sequence motif is also present in putative PSD homologs of other kinetoplastids, including Leishmania major (LmjF.35.4590) and Crithidia fasciculata (CFAC1.30097400). The predicted proteolytic cleavage motif of TbPSD is identical to that of P. falciparum PSD, rather than the typical GST motif of higher eukaryotes (Dowhan, 1997; Voelker, 1997; reviewed by Schuiki and Daum, 2009). To analyze whether TbPSD is proteolytically processed in vivo, we expressed a C-terminally 3xHA-tagged copy of Tb927.9.10080 in T. brucei procyclic and bloodstream forms. Analysis by SDS-PAGE followed by immunoblotting revealed two bands corresponding to the expected sizes of the tagged proenzyme (i.e. before proteolytic processing) and the α-subunit after proteolytic cleavage at the predicted recognition site (Fig. 3B). To further study whether the GSS motif of TbPSD is the cleavage site, we expressed a C-terminally 3xHA-tagged copy of TbPSD in which Ser318 was replaced by alanine. As shown in Fig. 3, changing
the cleavage site from GSS to GAS inhibited processing of the proenzyme, confirming the GSS motif as proteolytic cleavage site of TbPSD.

**TbPSD localizes to the mitochondrion**

Analysis by immunofluorescence microscopy showed good co-localization of TbPSD-3xHA with MitoTracker, a mitochondrial membrane potential-dependent red-fluorescent dye, in both procyclic and bloodstream forms (Fig. 4). Co-staining with other organelle markers (TbGRASP for Golgi, BiP for ER, Trypanopain for lysosome) showed no co-localization with TbPSD (results not shown). Mitochondrial localization was also observed for the uncleaved S318A TbPSD-3xHA mutant (Fig. 4), demonstrating that correct localization of TbPSD is independent of proteolytic processing of the proenzyme into its subunits.

**Down-regulation of TbPSD inhibits trypanosome growth**

Essentiality of TbPSD for *T. brucei* parasites in culture was studied by tetracycline-inducible RNAi-mediated gene silencing. Down-regulation of TbPSD mRNA was analyzed by Northern blotting after 3 days of RNAi induction and showed efficient depletion of TbPSD mRNA in both procyclic and bloodstream form parasites (Fig. 5A and B). After 4 days of RNAi against TbPSD, procyclic forms showed a growth defect compared to control parasites (Fig. 5A), while growth of TbPSD-depleted bloodstream forms was only slightly affected (Fig. 5B).

**TbPSD depletion affects mitochondrial integrity and ATP production via oxidative phosphorylation**

To study possible effects of TbPSD depletion on mitochondrial integrity, procyclic and bloodstream form trypanosomes were analyzed by fluorescence microscopy. Mitochondria were stained using two different markers: the membrane potential-dependent dye MitoTracker Red and an antibody against mitochondrial heat shock protein 70 (mtHSP70). While control cells showed the typical branched mitochondrial network in procyclic forms and the tubular mitochondrial structure in bloodstream
forms, trypanosomes depleted of TbPSD after 6 days of down-regulation revealed abnormal staining (Fig. 6A and B). In both life cycle forms, down-regulation of TbPSD resulted in the appearance of distinct and brightly fluorescent spots, an indication of mitochondrial fragmentation.

Subsequently, we measured mitochondrial ATP production in control and TbPSD-depleted procyclic forms in vitro using digitonin-solubilized membranes. Succinate was used as substrate to measure ATP formation via oxidative phosphorylation whereas 2-ketoglutarate was used to determine ATP synthesis via substrate level phosphorylation (Allemann and Schneider, 2000). The results showed a decrease in ATP production via oxidative phosphorylation of more than 75% in TbPSD-depleted mitochondria isolated from parasites after 6 days of RNAi compared to controls (Fig. 6C). In contrast, ATP production via substrate level phosphorylation was not affected (Fig. 6D).

**PE and PS levels are not affected by down-regulation of TbPSD**

It has previously been suggested that decarboxylation of PS may contribute to PE formation in *T. brucei* procyclic forms (Signorell et al., 2008b). The availability of RNAi parasites against TbPSD now allowed us to experimentally address the importance of this pathway in both procyclic and bloodstream forms. As expected, [3H]serine labeling of myriocin-treated parasites resulted in formation of [3H]PS and [3H]PE (see also Fig. 2). However, in contrast to our prediction that [3H]PE is generated by decarboxylation of [3H]PS, we saw no significant difference in [3H]PE formation in parasites after down-regulation of TbPSD (Fig. 7A). The ratios [3H]PS/[3H]PE and [3H]PE/[3H]PS in parasites after RNAi against TbPSD were unchanged compared to control uninduced cells (5.5 ± 1.3 vs. 6.0 ± 1.0 and 15.6 ± 2.8 vs. 14.5 ± 2.0 respectively; mean values ± standard deviations from three independent experiments). In addition, we saw no major differences in total phospholipid composition between control and TbPSD-depleted parasites after analysis of lipid extracts by two-dimensional TLC and phosphorus quantification (Supporting Information Fig. S4). Based on our findings that RNAi against TbPSD induces changes in mitochondrial structure, affects ATP production (Fig. 4) and reduces procyclic parasite growth (Fig. 5), we think it is unlikely that [3H]PE formation in TbPSD-depleted cells is due to residual amounts of TbPSD activity in these parasites. Alternatively, we hypothesize that [3H]PE may be formed by a reaction sequence that is independent from TbPSD, i.e. via decarboxylation of [3H]serine to [3H]ethanolamine followed by synthesis of [3H]PE by the Kennedy pathway. Such an interpretation is in line with the above-mentioned observation that digitonin-solubilized membranes readily form [3H]PS in vitro during incubation with [3H]serine, but are unable to convert [3H]PS to [3H]PE by mitochondrial TbPSD (Fig. 2C and D).

Interestingly, we observed that the amounts of [3H]PS formed during labeling with [3H]serine decreased during
a subsequent 4 h chase in the absence of [3H]serine (Fig. 7B). In contrast, labeling of [3H]PE remained unchanged during the chase (Fig. 7B), resulting in a change in the [3H]PS/[3H]PE ratio from 2.61 at the end of the pulse to 1.15 after the chase period (mean values from five independent experiments). As a similar change in the [3H]PS/[3H]PE ratio was also observed in TbPSD-depleted parasites, it is unlikely caused by TbPSD-mediated conversion of [3H]PS to [3H]PE. Alternatively, the loss of [3H]PS during the chase may be due to TbPSS2-mediated conversion of [3H]PS to PE, with concomitant loss of the label.

Together, [3H]serine-labeling experiments of TbPSD RNAi parasites and digitonin-solubilized membranes...
demonstrated that in *T. brucei* [³H]PE is not formed from [³H]serine via [³H]PS and subsequent decarboxylation to [³H]PE, but instead involves a previously unrecognized reaction sequence. Since plant cells (Rontein *et al.*, 2001) and *Plasmodium* parasites (Elabbadi *et al.*, 1997) are capable of decarboxylating serine to ethanolamine via serine decarboxylase, we investigated if a putative *T. brucei* serine decarboxylase activity may convert [³H]serine to [³H]ethanolamine, which would then enter the Kennedy pathway to form [³H]PE. However, using previously published protocols (Elabbadi *et al.*, 1997), we were unable to detect such an activity in *T. brucei* procyclic forms. In addition, we found no candidate gene in the *T. brucei* genome with homology to plant serine decarboxylase. It should be noted that the gene responsible for the observed serine decarboxylase activity in *Plasmodium* has not been reported.

**TbPSD is functional in E. coli**

To study whether Tb927.9.10080 encodes a functional PS decarboxylase, the full-length form was recombinantly expressed in *E. coli*. [³H]PS was then added to a membrane preparation and incubated for 10 min. Subsequently, lipids were extracted and separated by HPTLC and radiolabeled lipids visualized by fluorography. As seen in Fig. 8A, in the presence of either no vector or empty vector, there is some conversion of [³H]PS to [³H]PE, due to the endogenous *E. coli* PSD activity. In the presence of expressed TbPSD and *S. cerevisiae* PSD within this membrane background all of the [³H]PS is converted to [³H]PE, suggesting additional PSD activity that can be attributed to the expressed PSD homologs. To assay recombinant TbPSD in the absence of endogenous *E. coli* PSD, we expressed and purified a soluble form of TbPSD lacking the N-terminal membrane domain in *E. coli*. As shown in Fig. 8B, soluble truncated TbPSD was active and readily converted [³H]PS to [³H]PE in a concentration-dependent way. Together, these results demonstrate that TbPSD is active when expressed in *E. coli*.

**TbPSD is not functional in S. cerevisiae**

To test whether TbPSD enzyme is expressed and functional in yeast, multiple constructs of TbPSDs controlled...
processing of the pro-enzyme into its active form. Together, these results indicate that TbPSD constructs were not functional in yeast because they were poorly expressed and not effectively processed.

**Discussion**

Although the multiple pathways for PE biosynthesis are conserved among organisms, their contributions vary significantly. Several eukaryotes and most prokaryotes use decarboxylation of PS as main route for PE synthesis, while mammalian cells use both PS decarboxylation and the CDP-ethanolamine branch of the Kennedy pathway for PE formation. *T. brucei* parasites are unique in that the Kennedy pathway represents the only pathway for *de novo* production of PE (reviewed by Farine and Bütkofer, 2013). Its disruption, by depletion of any of the four enzymes catalyzing the individual reactions, leads to inhibition of growth and parasite death (Gibellini et al., 2008; Signorell et al., 2008b; Gibellini et al., 2009; Signorell et al., 2009). The presence of PS decarboxylase and PS synthase activities in *T. brucei* has been suggested in several reports (Menon et al., 1993; Ritkin et al., 1995; Signorell et al., 2008b), but TbPSD activity was later shown to be absent in bloodstream trypanosomes (Gibellini et al., 2009; Richmond et al., 2010). However, the enzymes were not characterized biochemically and the contributions of these pathways to PE formation and their importance for parasite viability were not investigated. We now report the identification and characterization of TbPSS2 and TbPSD. By using tetracycline-inducible RNAi against the individual enzymes, we demonstrate that their expression in *T. brucei* is essential for normal parasite growth in culture.

Our results using *in vivo* and *in vitro* [3H]serine labeling demonstrate that depletion of TbPSS2 in procyclic form trypanosomes completely blocks *de novo* formation of PS and reduces cellular PS levels, identifying TbPSS2 as the sole route for PS synthesis in *T. brucei*. PSS enzymes belong to two categories: in bacteria and yeast, PSS catalyzes PS formation using serine and CDP-DAG as substrates, whereas in mammals and plants PS is formed via head group exchange (reviewed by Farine and Bütkofer, 2013). Thus, our identification of a base exchange enzyme in the ancient eukaryote *T. brucei* is remarkable and is in accordance with recent results from *T. brucei* CDP-DAG knockout trypanosomes showing that PS production is largely unaffected after knocking down CDP-DAG synthesis (Lilley et al., 2014). Comparisons of the primary sequence of TbPSS2 with its predicted homologs in other kinetoplastids, including *T. cruzi* and Leishmania spp., indicate that PS synthesis in these parasites is
also catalyzed by a head group exchange enzyme (Ramakrishnan et al., 2013). Mammalian PSS1/2 enzymes have been localized to mitochondria-associated membranes (MAMs) (Stone and Vance, 2000). Enzyme activities were found in the ER and enriched in MAMs, while immunofluorescence studies using c-myc-tagged PSS1/2 showed colocalization with an ER marker (Stone and Vance, 2000). Consistent with the results in mammalian cells, using immunofluorescence microscopy we localized a C-terminally 3x-myc-tagged copy of TbPSS2, which was shown to be catalytically active, in the ER of T. brucei procyclic forms. At present, it is not known whether MAMs are also present in T. brucei parasites.

Incubation of T. brucei parasites with [3H]serine has been shown to not only label sphingolipids and PS, but also PE (Menon et al., 1993; Signorell et al., 2008b). Conversion of PS to PE has been observed in many organisms before and has been attributed to PSD enzymes (Kanfer and Kennedy, 1964; Butler and Morell, 1983; Vance and Vance, 1986; Trotter and Voelker, 1995; Elabbadi et al., 1997; Choi et al., 2012). Our results demonstrate that T. brucei parasites express a mitochondrial type I PSD enzyme, TbPSD, which undergoes similar proteolytic processing as its mammalian (Kuge et al., 1996) and yeast (Horvath et al., 2012) homologs. RNAi-mediated down-regulation of TbPSD affected mitochondrial morphology in both procyclic and bloodstream forms, reduced ATP production via oxidative phosphorylation in procyclic trypanosomes and reduced parasite growth in culture. When expressed in E. coli, but not in S. cerevisiae, TbPSD was active. However, as reported before for T. brucei bloodstream forms (Gibellini et al., 2009), we were unable to demonstrate in vitro or in vivo PS decarboxylation activity in procyclic forms. Although labeling of parasites with [3H]serine always leads to production of radiolabeled PE, suggesting the presence of a PS decarboxylation activity converting [3H]PS to [3H]PE, the reaction was not decreased in parasites after depletion of TbPSD. In addition, a mitochondrial extract competent for synthesizing [3H]PS was unable to generate [3H]PE. Together, these results indicate that using [3H]serine as substrate, [3H]PE in T. brucei is unlikely formed via PS followed by its decarboxylation to PE, but by a different mechanism involving soluble factors that are lost upon extraction of parasites with digitonin. The presence of a serine decarboxylase activity in T. brucei could explain our findings, however, we were unable to detect such an activity using published procedures (Elabbadi et al., 1997). In addition, mining the T. brucei predicted proteome with plant-type serine decarboxylases revealed no reasonable sequences. Since TbPSD is essential in T. brucei, we hypothesize that it may be involved in limited and locally restricted synthesis of PE in the mitochondrion, perhaps by producing a subset of PE molecular species important for mitochondrial structure and function, whereas bulk mitochondrial PE may be generated via the Kennedy pathway. Alternatively, we can’t exclude the possibility that TbPSD acts on a different substrate from PS, such as phosphatidylethanolamine as recently identified in T. gondii (Arroyo-Olarte et al., 2015).

**Experimental procedures**

Unless otherwise stated, all reagents were of analytical grade and purchased from Sigma-Aldrich (Buchs, Switzerland) or Merck (Darmstadt, Germany). Restriction enzymes were obtained from Fermentas (St. Leon-Rot, Germany) and antibiotics from Sigma-Aldrich, Invivogen (Nunningen, Switzerland) or Invitrogen (Basel, Switzerland). [3H]Serine (1 mCi ml⁻¹, 20 Ci mmol⁻¹) was purchased from American Radiolabeled Chemicals (St. Louis, USA) and [γ-32P]-dCTP (3000 Ci mmol⁻¹) from PerkinElmer (Waltham, USA). Phospholipid standards were purchased from Sigma-Aldrich or Avanti Polar Lipids Inc. (Alabaster, AL).

**Trypanosomes and culture conditions**

T. brucei procyclic forms expressing a T7 polymerase and a tetracycline repressor (obtained from Paul Englund, Baltimore) (Wirtz et al., 1999) were cultured at 27°C in SDM-79 (Brun and Schonenberger, 1979) containing 10% heat-inactivated fetal bovine serum (FBS, In Vitrogen), 25 μg ml⁻¹ hygromycin and 15 μg ml⁻¹ G418. Bloodstream New York single-marker trypanosomes were maintained at 37°C, 5% CO₂ in HMI-9 containing 10% FBS and 1 μg ml⁻¹ G418.

**Generation of 3xHA-tagged TbPSD and 3xc-myc-tagged TbPSS2**

To generate C-terminally 3xHA-tagged TbPSD (Tb927.9.10080), the corresponding open reading frame (ORF) was amplified by polymerase chain reaction (PCR) using primers PSD_HA_Fw GCCCAAGTTATGCTCGTCAACAGCAAACTGC and PSD_HA_Rv CGGCCTAAGCTTGCACCTCTCCGCACACAGCG (restriction sites underlined). The PCR product was ligated into the HindIII- and Xhol-digested plasmid pAG3020-3 (Gonzalez-Salgado et al., 2012) resulting in plasmid pLF1610HA. To produce N-terminally 3xc-myc-tagged TbPSS2, the corresponding ORF (Tb927.7.3760) was amplified using primers PSS2pJM2_Fw GCCGTCGAGGAGCGTAGCTGATACAGTACCG and PSS2pJM2_Rv CGGGTGATCTATCCGCACACGAAAGATATTAGCGTCTGGTAT and ligated into plasmid pJM2 (Desy et al., 2012), resulting in plasmid pLF3760cmyc. Before transfection into trypanosomes, plasmids were linearized using NotI enzyme to allow proper integration into the genome of the parasite.

**RNAi-mediated gene silencing**

Putative TbPSD mRNA was down-regulated in procyclic and bloodstream trypanosomes by RNAi-mediated gene
silencing using stem-loop constructs containing a puromycin resistance. A 374 bp fragment containing the last 361 bp of the TbPSD ORF and the first 13 bp of the TbPSD 3’UTR was amplified by PCR using primers PSD RNAi_Fw GCGCCCAAGCTGGATCCCTCTGCTCCTCCATTAAAGC and PSD RNAi_Rv TCTAGGCTCTAGCTAGTTGGCGACTGCTGACATCTGCCTACTTG (restriction sites underlined) and cloned into the tetracycline-inducible vector pMS14 (Serricchio and Bütkofer, 2013) resulting in plasmid pLF1610RNAi. For PSS2 RNAi in procyclic forms, a 550 bp fragment of the TbPS2 ORF was amplified using primers fwd3760 GTGAAGCTTGATCCCTCTGCTCCTCCATTAAAGC and rev3760 GTGAAGCTTGATCCCTCTGCTCCTCCATTAAAGC and cloned into the tetracycline-inducible vector pALC14 resulting in plasmid pJJ3760RNAi. Selection of the gene sequence for RNAi was done with RNAit, a prediction algorithm designed to prevent potential cross-talk and hence off-target effects (Redmond et al., 2003). Before transfection into trypanosomes, plasmids were linearized using NotI enzyme.

**Stable transfection of trypanosomes**

Trypanosomes were harvested at mid-log phase, washed once in phosphate-buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium phosphate, 2 mM monopotassium phosphate, pH 7.4) and suspended in 100 µl TBS buffer (90 mM sodium phosphate, 5 mM potassium chloride, pH 7.3) (Schumann Burkard et al., 2011) previously mixed with 10 µg of linearized plasmid (pLF1610HA, pLF1610RNAi, pJJ3760RNAi and pLF3760cmyc). Electroporation was performed in 100 µl Nucleocuvette using Lonza Nucleofector System (pulse code FI-115, “Primary Cell P3” solution). Recombinant clones were obtained by limited dilutions and selected with 1.75 µg ml⁻¹ phosphocillin for pLF1610RNAi or 2 µg ml⁻¹ puromycin for the other vectors. Proper integration of the constructs was confirmed by PCR using primers binding upstream of the recombination sites and at the end of the inserted genes. Expression of tagged proteins or generation of double-stranded RNA was induced by addition of 1 µg ml⁻¹ tetracycline to the culture medium.

**Northern blot analysis**

Total RNA was extracted from 4 x 10⁷ procyclic or bloodstream form trypanosomes using the Total SV RNA extraction Kit (Promega). 20 µg of RNA were loaded and run on a 1% agarose gel. The gel was stained with ethidium bromide and RNA amounts were assessed as loading control. After transfer onto a Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech) using 10x SSC buffer (150 mM trisodium citrate, pH 7.0, containing 1.5 M sodium chloride), RNA was cross-linked by UV irradiation. The membrane was probed with a [³²P]-labeled 374 bp probe of the TbPSD ORF/3’UTR or a [³²P]-labeled 550 bp probe of the TbPSS2 ORF, generated using the Prime-a-Gene labeling system (Promega). Detection was done by autoradiography using BioMax MS films (GE Healthcare, Buckinghamshire, UK) in combination with intensifying screens.

(Immuno-) Fluorescence microscopy

For MitoTracker staining, 1 x 10⁷ procyclic or 2 x 10⁶ bloodstream form trypanosomes were incubated for 30 min with 250 mM MitoTracker Red CM-H₂XRos (Invitrogen) in culture medium. After washing, parasites were spread and allowed to adhere to a microscopy slide, fixed with 4% paraformaldehyde, air dried and mounted with Vectashield containing DAPI (4’,6’-diamidino-2-phenylindole, Vector Laboratories). Alternatively, MitoTracker-stained and paraformaldehyde-fixed cells were used for co-localization studies using the following immunofluorescence protocol.

For immunofluorescence microscopy, trypanosomes were collected and washed before being allowed to adhere onto a microscopy slide (Thermo Scientific) for 10 min. Cells were fixed with 4% paraformaldehyde, washed 3× with cold PBS and permeabilized with 0.2% Triton X-100 in PBS. Blocking was performed with 2% bovine serum albumin in PBS for 30 min followed by 30 min incubation of first antibody diluted in blocking solution. The following antibodies were used: mouse monoclonal α-HA, 1:16 1212 (Covance, 1:250 dilution), mouse α-c-myc (Santa Cruz Biotech, 1:200 dilution) or rabbit α-c-myc (Bethyl Laboratories, 1:200 dilution), mouse α-mtHSP70 (kindly provided by Paul Englund, Baltimore, 1:1000 dilution) and rabbit α-BiP (kindly provided by Jay Bangs, Buffalo, 1:2000 dilution). After washing, secondary antibodies goat anti-mouse and anti-rabbit AlexaFluor 594 and 488 (Invitrogen, 1:800 dilution in blocking solution) were added for 45 min in either combinations. After washing and air-drying, cells were mounted with Vectashield containing DAPI. Fluorescence microscopy was performed with a Leica DMi6000 B inverted microscope using a 60× oil objective. Pictures were acquired, processed and 3D-deconvolved with the Leica LAS AF Version 2.1.0 software (Leica Microsystems CMS GmbH).

**SDS-PAGE and immunoblotting**

Extracted proteins were separated on 12% polyacrylamide gels under reducing conditions (Laemmli, 1970). Proteins were transferred on Immobilon-P polyvinylidene fluoride membranes (Millipore, USA) by semi-dry blotting. After blocking in TBS buffer (10 mM Tris-HCl pH 7.5, 144 mM NaCl) containing 5% (wt/vol) milk powder, membranes were exposed to α-HA mouse monoclonal primary antibody (1:3000). Horseradish peroxidase-conjugated secondary antibody anti-mouse (Dako, Agilent Technologies) was used at a concentration of 1:5000 and detected using an enhanced chemiluminescence detection kit (Pierce).

[^H]Serine labeling and lipid analysis

Procyclic form trypanosomes (5–10 x 10⁷) were pre-treated with 2.5 mM myriocin for 30 min and labeled with 30 µCi[^H]serine for 4 h. For pulse/chase experiments, parasites labeled for 4 h were collected, washed once and resuspended in fresh medium without radioactivity. The chase was conducted for 2 to 6 h. Lipids were extracted according to the work by Bligh and Dyer (1959) and separated by one-dimensional thin layer chromatography (TLC) (Silica gel 60
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Preparation and labeling of membranes-containing digitonin extracts

Digitonin extracts were prepared as described elsewhere (Schneider et al., 2007). Procylic form trypanosomes (1 × 10⁶ cells) were harvested, washed once in SBG buffer (150 mM Tris-HCl pH 7.9, 20 mM glucose, 20 mM NaH₂PO₄) and suspended in SoTE buffer (20 mM Tris-HCl pH 7.5, with 600 mM sorbitol and 2 mM EDTA) containing a final concentration of 0.025% (wt/vol) digitonin. After 5 min of incubation on ice, mitochondria were isolated by centrifugation at 6200 g. After removal of the supernatant, membranes from digitonin extract were resuspended in 500 µl SoTE and labeled with 15C [³H]serine (ARC, specific activity 30.5 Ci mmol⁻¹) with or without 300 µM substrate (PE, PS, PI, DAG, CDP-DAG; all dipalmitoyl) in a final volume of 200 µl. After sonication reactions were incubated at 30°C for 1 h, and terminated by the addition of 750 µl methanol: chloroform (2:1, by vol). Lipids were extracted in the organic lower layer after making the extract biphasic by the addition of 250 µl of water and chloroform, allowing the incorporation of the radiolabel to be quantitated by liquid scintillation counting.

ATP production assay

ATP production in digitonin extracts was measured as previously described (Allemann and Schneider, 2000). About 5 mM of succinate or 2-ketoglutarate and 67 µM of ADP were added to membranes isolated by digitonin extraction from 9.5 × 10⁷ procylic form trypanosomes and incubated for 30 min at room temperature. Antimycin (inhibitor of complex III) and atracyloside (inhibitor of ADP/ATP translocase) were pre-incubated with digitonin extracts for 10 min at a concentration of 2.7 and 43 µM, respectively. ATP concentrations were determined using ATP Bioluminescence Assay Kit CLS II (Roche, Basel, Switzerland).

Recombinant expression and enzyme activity of TbPSS2 in E. coli

The TbPSS2 ORF was obtained by PCR from T. brucei gDNA, using 5'-CGGGGATCCGGATGCGGCGGATACGCTCAA C-3' and 5'-CCGCTCGAGTCGCCAAGATTATGTA-3' primers. The PCR product was sub-cloned into pET32b expression vector, expression was induced in E. coli (BL-21 strain) at 15°C overnight with 0.5 mM isopropyl β-D-1-thiogalactopyranoside. E. coli membranes were isolated and PSS activity assays conducted, using an empty vector expressing E. coli as a control. PSS activity was assayed as follows. 100 mM potassium phosphate (pH 7, 5), 20 mM MgCl₂, 1% (wt/vol) Triton X-100, 50 µg membrane protein and 0.05 µCi [³H]serine (ARC, specific activity 30.5 Ci mmol⁻¹) with or without 300 µM substrate (PE, PS, PI, DAG, CDP-DAG; all dipalmitoyl) in a final volume of 200 µl. After sonication reactions were incubated at 30°C for 1 h, and terminated by the addition of 750 µl methanol: chloroform (2:1, by vol). Lipids were extracted in the organic lower layer after making the extract biphasic by the addition of 250 µl of water and chloroform, allowing the incorporation of the radiolabel to be quantitated by liquid scintillation counting.

TbPSSD activity assay in E. coli

The TbPSSD and ScPSSD ORF were obtained by PCR from gDNA and cDNA respectively, using the TbPSSD primers 5'-CCGGGATCCGGATGCGGCGGATACGCTCAA C-3' (forward for full length TbPSSD) or 5'-CCGGGATCCGGGATACGCT CAACTTGGCAGCA-3' (forward for truncated TbPSSD) and 5'-CCGCTCGAGCTTGCCACTTCTCGACAA-3' (reverse), while for the ScPSSD ORF 5'-CCGGGATCCGGATGCTTCAAT ATGCCAGTTAAGAAGC-3' and 5'-CCGCTCGAGTGGTTTAATA ATATTTCCTGGATTA-3' primers were used. The PCR products were digested and ligated into pET 32b using BamH I and XhoI. TbPSSD expression was induced with IPTG (0.05 mM) in BL21 (DE3) at 15°C overnight and confirmed by immunoblot analysis. The full length PSSD assay utilized a membrane preparation (100 µg membrane protein) from either BL-21 cells only or with empty pET 32b or pET 32b-TbPSSD or pET 32b-ScPSSD. Assay includes 100 mM Hepes (pH 7, 4), 20 mM MgCl₂, 0.3% n-Octyl-glucopyranoside and 0.1 µCi [³H]PSS (specific activity 60 Ci mmol⁻¹). Reactions were performed at 30°C for 10 min. After termination, lipids were extracted and separated on high performance thin layer chromatography silica plates in chloroform:methanol:water (65:25:4, by vol), radioimaged PS and PE were detected by fluorography. The truncated soluble form of TbPSSD was expressed in the same manner as the full length enzyme but then purified using Ni²⁺ affinity purification. Various amounts of protein were assayed in the same way as above except reactions were performed at 30°C for 60 min.

Construction of TbPSSD vectors and complementation of the yeast psd1Δpsd2Δdpl1Δ strain

Constitutive expression vectors harboring a full length TbPSSD and truncated TbPSSDs (lacking the first 48 or 83 amino acids) were made using the pBEVY vector (Miller, 1998). Briefly, specific primers for the individual constructs were generated and used to amplify DNA from pALC14 vector harboring TbPSSD using PCR with Phusion High-Fidelity DNA polymerase (New England Biolabs Inc.).
**Phosphatidylserine metabolism of T. brucei**

Phosphatidylserine metabolism of T. brucei involves the production of phosphatidyl-serine by the parasite, which is essential for the maintenance of the parasite's membrane integrity. The authors describe the construction and characterization of a non-mitochondrial type I phosphatidyl-serine decarboxylase in Plasmodium falciparum. The enzyme activity is measured using a radiometric assay and its activity is shown to be dependent on the presence of certain lipids and proteins.

**References**

Allemann, N., and Schneider, A. (2000) ATP production in isolated mitochondria of procyclic Trypanosoma brucei. *Mol Biochem Parasitol* 111: 87–94.

Arroyo-Olarte, R.D., Brouwers, J.F., Kuchipudi, A., Helms, J.B., Biswas, A., Dunay, I.R., et al. (2015) Phosphatidyl-threonine and lipid-mediated control of parasite virulence. *PLoS Biol* 13: e1002288.

Bannai, H., Tamada, Y., Maruyama, O., Nakai, K., and Miyano, S. (2002) Extensive feature detection of N-terminal protein sorting signals. *Bioinformatics* 18: 298–305.

Baunaure, F., Eldin, P., Cathiard, A.M., and Vial, H. (2003) Characterization of a non-mitochondrial type I phosphatidyl-serine decarboxylase in *Plasmodium falciparum*. *Mol Microbiol* 51: 33–46.

Bevers, E.M., Comfurius, P., and Zwaal, R.F. (1983) Changes in membrane phospholipid distribution during platelet activation. *Biochim Biophys Acta* 736: 57–66.

Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37: 911–917.

Bogdanov, M., and Dowhan, W. (1998) Phospholipid-assisted protein folding: phosphatidylethanolamine is required at a late step of the conformational maturation of the polytopic membrane protein lactose permease. *EMBO J* 17: 5255–5264.

Borkenhagen, L.F., Kennedy, E.P., and Fielding, L. (1961) Phospholipase A activity in human brain and its role in the metabolism of phospholipids. *J Neurochem* 41: 1445–1454.

Choi, J.Y., Augagneur, Y., Ben Mamoun, C., and Voelker, D.R. (2012) Identification of gene encoding *Plasmodium knowlesi* phosphatidylserine decarboxylase by genetic complementation in yeast and characterization of in vitro maturation of encoded enzyme. *J Biol Chem* 287: 222–232.

Cullen, T.W., and Trent, M.S. (2010) A link between the assembly of flagella and lipopolysaccharide of the Gram-negative bacterium *Campylobacter jejuni*. *Proc Natl Acad Sci USA* 107: 5160–5165.
Desy, S., Schneider, A., and Mani, J. (2012) Trypanosoma brucei has a canonical mitochondrial processing peptidase. Mol Biochem Parasitol 185: 161–164.

Dowhan, W. (1997) Phosphatidylserine decarboxylases: pyruvoyl-dependent enzymes from bacteria to mammals. Methods Enzymol 280: 81–88.

Elabbadi, N., Ancelin, M.L., and Vial, H.J. (1997) Phospholipid metabolism of serine in Plasmodium-infected erythrocytes involves phosphatidylserine and direct serine decarboxylation. Biochem J 324: 435–445.

Emoto, K., Kobayashi, T., Yamaji, A., Aizawa, H., Yahara, I., Inoue, K., and Umeda, M. (1996) Redistribution of phosphatidylethanolamine at the cleavage furrow of dividing cells during cytokinesis. Proc Natl Acad Sci USA 93: 12867–12872.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., and Henson, P.M. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 148: 2207–2216.

Farine, L., and Büttikofer, P. (2013) The ins and outs of phosphatidylethanolamine synthesis in Trypanosoma brucei. Biochim Biophys Acta 1831: 533–542.

Fevre, E.M., Wissmann, B.V., Welburn, S.C., Lutumba, P., and Fèvre, E.M. (2008) The burden of human African trypanosomiasis. PLoS Negl Trop Dis 2: e333.

Finn, R.D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R.Y., Eddy, S.R., et al. (2014) Pfam: the protein families database. Nucleic Acids Res 42: D222–D230.

Gibellini, F., and Smith, T.K. (2010) The Kennedy pathway – de novo synthesis of phosphatidylethanolamine and phosphatidylcholine. IUBMB Life 62: 414–428.

Gibellini, F., Hunter, W.N., and Smith, T.K. (2008) Biochemical characterization of the initial steps of the Kennedy pathway in Trypanosoma brucei: the ethanolamine and choline kinases. Biochem J 415: 135–144.

Gibellini, F., Hunter, W.N., and Smith, T.K. (2009) The ethanolamine branch of the Kennedy pathway is essential in the bloodstream form of Trypanosoma brucei. Mol Microbiol 73: 826–843.

Gonzalez-Salgado, A., Steinmann, M.E., Greganova, E., Rauch, M., Mäser, P., Sigel, E., and Büttikofer, P. (2012) Myo-inositol uptake is essential for bulk inositol phospholipid but not glycosylphosphatidylinositol synthesis in Trypanosoma brucei. J Biol Chem 287: 13313–13323.

Gupta, N., Hartmann, A., Lucius, R., and Voelker, D.R. (2012) The obligate intracellular parasite Toxoplasma gondii secretes a soluble phosphatidylserine decarboxylase. J Biol Chem 287: 22938–22947.

Horvath, S.E., Böttinger, L., Vögte, F.N., Wiedemann, N., Meisinger, C., Becker, T., and Daum, G. (2012) Processing and topology of the yeast mitochondrial phosphatidylserine decarboxylase 1. J Biol Chem 287: 36744–36755.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., et al. (2000) A ubiquitin-like system mediates protein lipidation. Nature 408: 488–492.

Kanfer, J., and Kennedy, E.P. (1964) Metabolism and function of bacterial lipids II. Biosynthesis of phospholipids in Escherichia coli. J Biol Chem 239: 1720–1726.

Kennedy, E.P., and Weiss, S.B. (1956) The function of cytidine coenzymes in the biosynthesis of phospholipides. J Biol Chem 222: 193–214.

Kitamura, H., Wu, W.I., and Voelker, D.R. (2002) The C2 domain of phosphatidylserine decarboxylase 2 is not required for catalysis but is essential for in vivo function. J Biol Chem 277: 33720–33726.

Kuge, O., Saito, K., Kojima, M., Akamatsu, Y., and Nishijima, M. (1996) Post-translational processing of the phosphatidylserine decarboxylase gene product in Chinese hamster ovary cells. Biochem J 319: 33–38.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lee, A.G. (2004) How lipids affect the activities of integral membrane proteins. Biochim Biophys Acta 1666: 62–87.

Lilley, A.C., Major, L., Young, S., Stark, M.J.R., and Smith, T.K. (2014) The essential roles of cytidine diphosphate-diacylglycerol synthase in bloodstream form Trypanosoma brucei. Mol Microbiol 92: 453–470.

Matsumoto, K. (1997) Phosphatidylserine synthase from bacteria. Biochim Biophys Acta 1348: 214–227.

Menon, A.K., and Stevens, V.L. (1992) Phosphatidylethanolamine is the donor of the ethanolamine residue linking a glycosylphosphatidylinositol anchor to protein. J Biol Chem 267: 15277–15280.

Menon, A.K., Eppinger, M., Mayor, S., and Schwarz, R.T. (1993) Phosphatidylethanolamine is the donor of the terminal phosphoethanolamine group in trypanosome glycosylphosphatidylinositol. EMBO J 12: 1907–1914.

Miller, C. (1998) Assessment of aryl hydrocarbon receptor complex interactions using pBEVY plasmids: expression vectors with bi-directional promoters for use in Saccharomyces cerevisiae. Nucleic Acids Res 26: 3577–3583.

Nerlich, A., Orlow, M., von Rontein, D., Hanson, A.D., Dörmann, P., and Dörmann, P. (2007) Deficiency in phosphatidylserine decarboxylase activity in the psd1 psd2 psd3 triple mutant of Arabidopsis affects phosphatidylethanolamine accumulation in mitochondria. Plant Physiol 144: 904–914.

Nikawa, J.I.I., and Yamashita, S. (1981) Characterization of phosphatidylserine synthase from Saccharomyces cerevisiae and a mutant defective in the enzyme. Biochim Biophys Acta 665: 420–426.

Ohta, A., and Shibuya, I. (1977) Membrane phospholipid synthesis and phenotypic correlation of an Escherichia coli psd mutant. J Bacteriol 132: 434–443.

van Poeije, P.D., and Snell, E.E. (1990) Pyruvoyl-dependent enzymes. Annu Rev Biochem 59: 29.

Ramakrishnan, S., Serricchio, M., Striepen, B., and Büttikofer, P. (2013) Lipid synthesis in protozoan parasites: a comparison between kinetoplastids and apicomplexans. Prog Lipid Res 52: 488–512.

Redmond, S., Vadivelu, J., and Field, M.C. (2003) RNAi: an automated web-based tool for the selection of RNAi targets in Trypanosoma brucei. Mol Biochem Parasitol 128: 115–118.

Richmond, G.S., Gibellini, F., Young, S.A., Major, L., Denton, H., Lilley, A., and Smith, T.K. (2010) Lipidomic analysis of bloodstream and procyclic form Trypanosoma brucei. Parasitology 137: 1357–1392.
Suzuki, T.T., and Kanfer, J.N. (1985) Purification and properties of an ethanolamine-serine base exchange enzyme of rat brain microsomes. *J Biol Chem* **260**: 1394–1399.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J Biol Chem* **254**: 3692–3695.

Trotter, P.J., and Voelker, D.R. (1995) Identification of a non-mitochondrial phosphatidylserine decarboxylase activity (PSD2) in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* **270**: 6062–6070.

Trotter, P.J., Pedretti, J., and Voelker, D.R. (1993) Phosphatidylserine decarboxylase from *Saccharomyces cerevisiae*. Isolation of mutants, cloning of the gene, and creation of a null allele. *J Biol Chem* **268**: 21416–21424.

Trotter, P.J., Pedretti, J., Yates, R., and Voelker, D.R. (1995) Phosphatidylserine decarboxylase 2 of *Saccharomyces cerevisiae*. Cloning and mapping of the gene, heterologous expression, and creation of the null allele. *J Biol Chem* **270**: 6071–6080.

Vance, J.E. (2008) Phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically related aminophospholipids. *J Lipid Res* **49**: 1377–1387.

Vance, J.E. (2014) MAM (mitochondria-associated membranes) in mammalian cells: lipids and beyond. *Biochim Biophys Acta* **1841**: 595–609.

Vance, J.E., and Steenbergen, R. (2005) Metabolism and functions of phosphatidylserine. *Prog Lipid Res* **44**: 207–234.

Vance, J.E., and Tasseva, G. (2013) Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. *Biochim Biophys Acta* **1831**: 543–554.

Vance, J.E., and Vance, D.E. (1986) Specific pools of phospholipids are used for lipoprotein secretion by cultured rat hepatocytes. *J Biol Chem* **261**: 4486–4491.

Vickerman, K. (1985) Developmental cycles and biology of pathogenic trypanosomes. *Br Med Bull* **41**: 105–114.

Voelker, D.R. (1997) Phosphatidylserine decarboxylase. *Biochim Biophys Acta* **1348**: 236–244.

Wirtz, E., Leal, S., Ochatt, C., and Cross, G.A. (1999) A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. *Mol Biochem Parasitol* **99**: 89–101.

Yamashita, S., and Nikawa, J. (1997) Phosphatidylserine synthase from yeast. *Biochim Biophys Acta* **1348**: 228–235.

Zhang, K., Pompey, J.M., Hsu, F.F., Key, P., Bandhuvula, P., Saba, J.D., et al. (2007) Redistribution of sphingolipid metabolism toward de novo synthesis of ethanolamine in Leishmania. *EMBO J* **26**: 1094–1104.

**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.