

# Sphingolipids are essential for differentiation but not growth in *Leishmania*

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**Sphingolipids (SLs) play critical roles in eukaryotic cells in the formation of lipid rafts, membrane trafficking, and signal transduction. Here we created a SL null mutant in the protozoan parasite *Leishmania major* through targeted deletion of the key *de novo* biosynthetic enzyme serine palmitoyltransferase subunit 2 (SPT2). Although SLs are typically essential, *spt2*<sup>-</sup> *Leishmania* were viable, yet were completely deficient in *de novo* sphingolipid synthesis, and lacked inositol phosphorylceramides and other SLs. Remarkably, *spt2*<sup>-</sup> parasites maintained ‘lipid rafts’ as defined by Triton X-100 detergent resistant membrane formation. Upon entry to stationary phase *spt2*<sup>-</sup> failed to differentiate to infective metacyclic parasites and died instead. Death occurred not by apoptosis or changes in metacyclic gene expression, but from catastrophic problems leading to accumulation of small vesicles characteristic of the multivesicular body/multivesicular tubule network. Stage specificity may reflect changes in membrane structure as well as elevated demands in vesicular trafficking required for parasite remodeling during differentiation. We suggest that SL-deficient *Leishmania* provide a useful biological setting for tests of essential SL enzymes in other organisms where SL perturbation is lethal.**

**Keywords:** gp63-major surface protease-leishmanolysin/lipophosphoglycan/sphingoid base synthesis/trypansomatid parasite/virulence

## Introduction

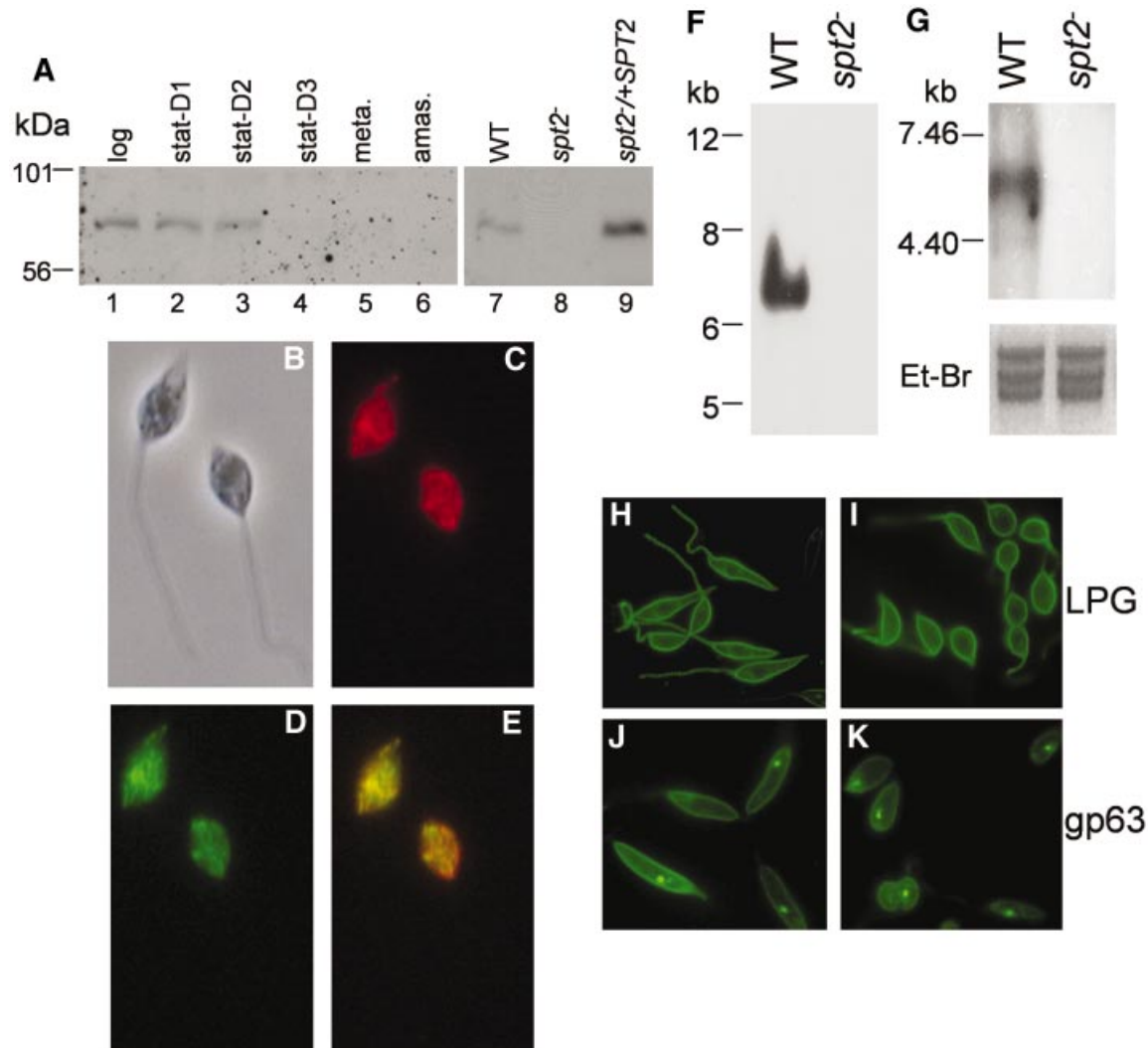
*Leishmania* are covered with a variety of interrelated glycosylphosphatidylinositol (GPI)-anchored molecules, including lipophosphoglycan (LPG), small glycosylinositolphospholipids (GIPLs), proteophosphoglycan (PPG) and GPI-anchored proteins. Numerous studies have suggested ways in which these molecules may play a role in steps important for *Leishmania* survival and pathogenesis (reviewed in Ilgoutz and McConville, 2001; Sacks and Kamhawi, 2001). These include survival in the sand fly vector, where the flagellated promastigote form differentiates from a replicating procyclic to a non-replicating, infectious metacyclic form, and in the mammalian host, where parasites ingested by macrophages differentiate

into the non-flagellated amastigotes that ultimately cause disease.

In contrast to GPI-anchored molecules, the roles of sphingolipids (SLs), a diverse family of ubiquitous eukaryotic membrane-anchored lipids, are largely unknown, although they comprise 5–10% of *Leishmania* membrane lipids (Kaneshiro *et al.*, 1986). SLs, ceramides and sphingoid bases (SBs) play crucial roles in general membrane function, cell-to-cell recognition, regulation of cell growth and differentiation, intracellular signaling, apoptosis and modulation of the immune response (Merrill *et al.*, 1993; Vaux and Korsmeyer, 1999; Kolter *et al.*, 2002; Maceyka *et al.*, 2002). Previous studies showed that *Leishmania* parasites synthesize inositol phosphorylceramide (IPC), which is absent in mammals but present in fungi and plants (Kaneshiro *et al.*, 1986; Lester and Dickson, 1993), and ceramide-anchored molecules in *Trypanosoma cruzi* may alter host cell signaling (Lederkremer and Bertello, 2001; DosReis *et al.*, 2002; McConville *et al.*, 2002).

SLs are enriched at key points within both exocytic and endocytic pathways and play important roles in trafficking (Gruenberg, 2001; Ikonen, 2001; Funato *et al.*, 2002). *Leishmania* and the related trypanosomes have an unusual polarized organization of secretory and degradative pathways, where endocytosis and exocytosis are restricted to a small invagination termed the ‘flagellar pocket’ (Landfear and Ignatushchenko, 2001). Macromolecular flux through this organelle is intense due to the demands associated with maintenance and remodeling of its surface during the infectious cycle, as well as from nutrient acquisition. Current knowledge about vesicular trafficking is relatively limited, and suggests that there are significant differences with other eukaryotes. One involves the multivesicular tubule (MVT), a terminal lysosome-like organelle (Ilgoutz *et al.*, 1999; Weise *et al.*, 2000; Ghedin *et al.*, 2001; Mullin *et al.*, 2001; Waller and McConville, 2002). The MVT contains vesicles of ~50 nm in size, which appear to arise from an upstream organelle resembling multivesicular bodies (MVBs) (Mullin *et al.*, 2001; Katzmann *et al.*, 2002; Waller and McConville, 2002). In mammalian cells, MVB-derived vesicles are rich in SLs and cholesterol, while the limiting membranes of the late endosome and MVB are poor in these lipids (Kobayashi *et al.*, 2002; Mobius *et al.*, 2003; Wubbolts *et al.*, 2003). In *Leishmania* exogenous ceramides accumulate in the MVT rather than in the Golgi (Ghedini *et al.*, 2001; Mullin *et al.*, 2001; Waller and McConville, 2002).

*De novo* SL biosynthesis starts with the condensation of L-serine and palmitoyl-CoA into 3-ketosphinganine (3-ketodihydrospingosine or 3-KDS). This is catalyzed by the pyridoxal 5'-phosphate-dependent enzyme serine palmitoyltransferase (SPT, EC 2.3.1.50), which is encoded by two subunits, *SPT1* and *SPT2* (reviewed by Hanada,



**Fig. 1.** Characterization of the *SPT2* gene and its expression in *L. major*. (A) Developmental expression of SPT2 protein. Whole cell lysates ( $1 \times 10^6$ ) from WT log promastigotes (lanes 1 and 7), WT stationary promastigotes (days 1–3, lanes 2–4), metacyclics (lane 5), WT amastigotes (lane 6), *spt2*<sup>-</sup> log promastigotes (lane 8) and *spt2*<sup>+/+</sup>*SPT2* log promastigotes (lane 9) were subjected to immunoblot analysis with affinity purified anti-SPT2 antibody. (B)–(E) GFP-SPT2 is localized to the ER. WT *L. major* cells were transfected with pXG-GFP-SPT2 and grown in the presence of 50  $\mu$ g/ml G418. (B) Phase contrast. (C) Immunofluorescent staining with rabbit anti-*T. brucei* BIP antibody followed by Texas Red-labeled goat anti-rabbit IgG. (D) GFP fluorescence of GFP-SPT2. (E) Merge of (C) and (D). (F) Southern blot analysis of genomic DNA from WT and *spt2*<sup>-</sup> digested with *Eco*RI. The probe was the entire *SPT2* ORF. (G) Northern blot analysis of total RNA from WT and *spt2*<sup>-</sup> null mutant. (H)–(K) Distribution of LPG and gp63 are unaltered in log phase *spt2*<sup>-</sup>. Localizations of LPG and gp63 in log phase WT (H and J, respectively) and *spt2*<sup>-</sup> (I and K) were determined by immunofluorescence microscopy.

2003). Deletion or inhibition of *SPT2* in yeast and mammalian cells leads to severe growth retardation and death (Pinto *et al.*, 1992a; Hanada *et al.*, 2000). Here we used targeted deletion of *Leishmania SPT2* to show that SLs are not required for growth in log phase, or formation of detergent resistant membranes (DRMs) associated with lipid ‘rafts’.

## Results

### Identification of genes encoding the subunits of *Leishmania major* serine palmitoyltransferase

Two ORFs with homology to the SPT subunits *SPT1* and *SPT2* were identified from the *L. major* genome project data. Each was recovered by PCR from genomic DNA and sequenced (GenBank AY235575, AY235574). The *SPT1*

and *SPT2* genes predicted proteins of 488 and 538 amino acids, which showed orthologous relationships to those of other eukaryotes (35–36% and 35–46% identity, respectively, for comparisons with human, yeast or *Arabidopsis*). *Leishmania major* *SPT2* contained the catalytic site motif (<sup>368</sup>GTFTKSSFG<sup>375</sup>), where K372 is thought to bind pyridoxal 5′-phosphate (reviewed by Hanada, 2003). Southern blot analysis of *L. major* genomic DNA showed that *SPT2* was single-copy (data not shown). Western blot analysis with anti-SPT2 antibody showed that SPT2 occurred at high levels in log and early stationary promastigotes, but was down-regulated in late stationary promastigotes, metacyclic and amastigote stages (Figure 1A, lanes 1–6). Similar data were obtained by northern blot analysis (data not shown). *Leishmania major* *SPT2* was localized to the endoplasmic reticulum, as

revealed by the similar distributions of a functional GFP-SPT2 fusion protein with the ER marker BIP (Figure 1B–E).

### Targeted replacement of *L. major* SPT2

To test the requirement for *de novo* SL synthesis in *Leishmania* we chose to delete the catalytic SPT2 subunit. Homologous gene replacement works efficiently in *Leishmania* although two rounds of targeting are necessary. Remarkably, *spt2*<sup>-</sup> colonies bearing homozygous  $\Delta spt2::HYG/\Delta spt2::PAC$  replacements were readily obtained, whether or not SBs were provided. Hybridizations with a SPT2 ORF probe confirmed the lack of the SPT2 gene and transcript in these lines (Figure 1F and G). As a control we reintroduced the SPT2 gene on an episomal *Leishmania* expression vector (pXG-SPT2), and this ‘add-back’ line is termed *spt2*<sup>-</sup>/+SPT2. This line showed overexpression of SPT2 (Figure 1A, lanes 7–9), without any apparent adverse effects.

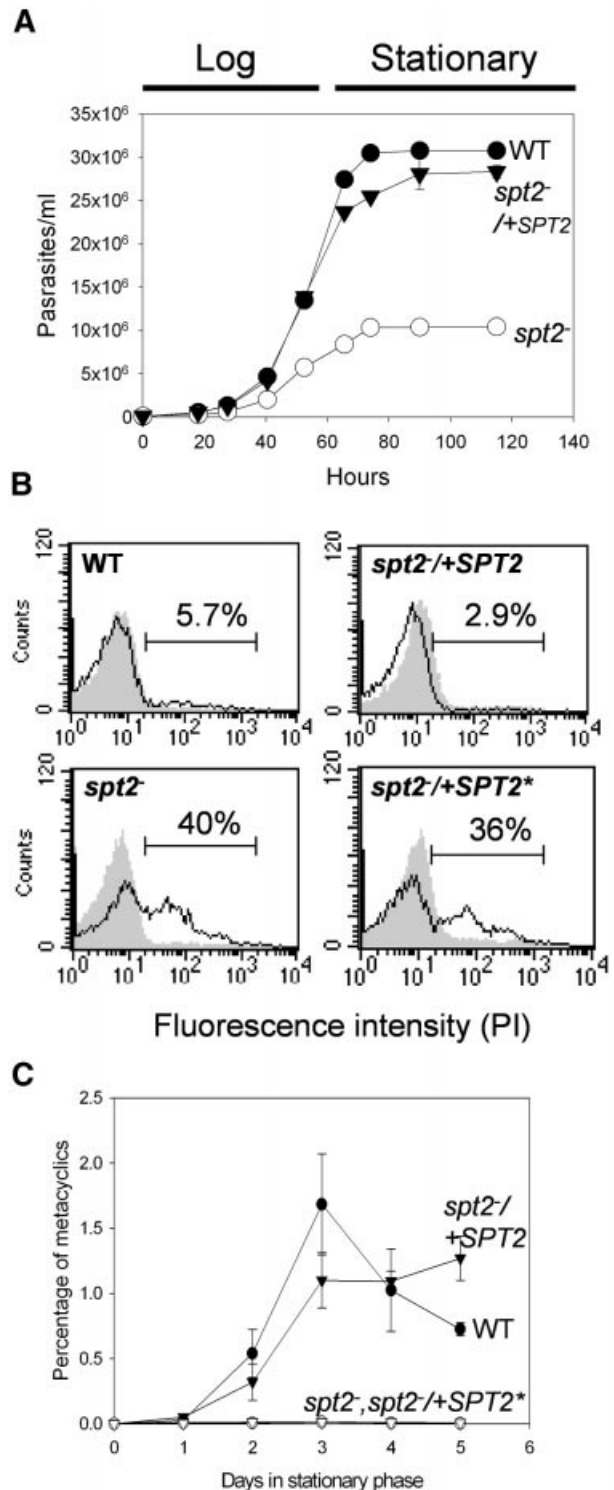
### *spt2*<sup>-</sup> promastigotes grow somewhat more slowly in log phase but crash in stationary phase and fail to generate infective metacyclics

In log phase, *spt2*<sup>-</sup> promastigotes grew somewhat more slowly than wild type (WT), with a doubling time of ~9.5 h versus 7.3 h, and attained lower densities in stationary phase (8–10 × 10<sup>6</sup>/ml versus 2.5–3.2 × 10<sup>7</sup>/ml; Figure 2A). During exponential growth both WT and *spt2*<sup>-</sup> showed good viability, as judged by dye exclusion (Figure 2B, shaded peaks). Immunofluorescence microscopy with antibody against LPG or the GPI-anchored protein gp63 showed normal surface expression in log-phase *spt2*<sup>-</sup>, although these cells appeared somewhat less elongate (Figure 1H–K).

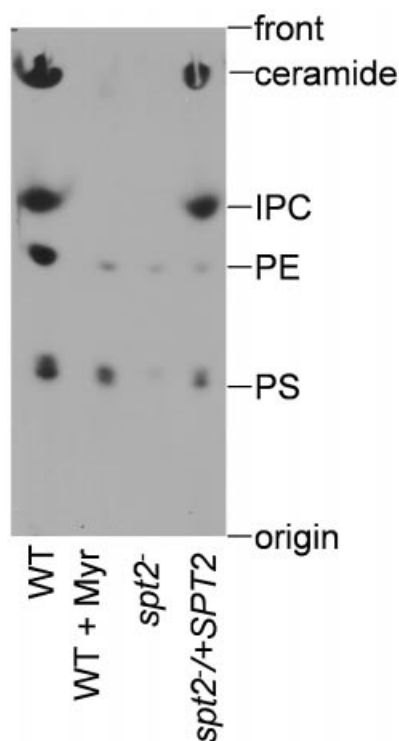
Coincident with entry into stationary phase, the *spt2*<sup>-</sup> parasites showed progressively higher frequencies of cell shape abnormalities and loss of viability (Figure 2B, unfilled peaks). After 3 days in stationary phase, 35–50% of the *spt2*<sup>-</sup> cells were permeable to propidium iodide (PI), versus 5.7% of WT (Figure 2B), consistent with microscopic evaluation (data not shown). Restoration of WT SPT2 but not a catalytic site mutant SPT2\* (K372A) expression in *spt2*<sup>-</sup> fully reversed this defect (Figure 2B, *spt2*<sup>-</sup>/+SPT2 and *spt2*<sup>-</sup>/+SPT2\*). Thus while not required in log phase, SPT2 played a vital role in stationary phase.

The stationary phase defect was particularly interesting, as cessation of growth marks the onset of differentiation to the infective metacyclic stage (Sacks and Perkins, 1984). Metacyclics are distinguished by morphology, reactivity

with lectins and/or monoclonal antibodies, sedimentation, expression of stage-specific genes such as the *HASP* and *SHERP* gene family, and infectivity (Knuepfer *et al.*, 2001; Sacks and Perkins, 1985; Späth and Beverley, 2001). By these criteria *spt2*<sup>-</sup> failed to produce metacyclics: e.g., WT metacyclics appeared after entry into stationary phase and peaked after 3–4 days (Figure 2C, WT), whereas few were found in the *spt2*<sup>-</sup> parasites at any point (<0.01%, Figure 2C, *spt2*<sup>-</sup>). Importantly, normal metacyclic levels returned when SPT2 but not SPT2\*



**Fig. 2.** Deletion of SPT2 results in slower growth during log phase, poor viability during stationary phase and failure to differentiate. (A) WT, *spt2*<sup>-</sup> or *spt2*<sup>-</sup>/+SPT2 were inoculated into M199 medium at  $2 \times 10^5$  cells/ml and cell densities were measured. Experiments were carried out in duplicate and error bars represent standard deviations. (B) Flow cytometry of WT, *spt2*<sup>-</sup>, *spt2*<sup>-</sup>/+SPT2 or *spt2*<sup>-</sup>/+SPT2\* (*spt2*<sup>-</sup> transfected with a modified SPT2 that contains a K372A mutation that abolishes the binding to pyridoxal 5'-phosphate: Supplementary data) labeled with 0.5 µg/ml of propidium iodide (PI). Shaded peaks represent log phase cells, and unfilled peaks indicate day 3 stationary phase cells (3 days after reaching stationary phase). The percentage of PI-positive cells in each stationary culture is shown. (C) *spt2*<sup>-</sup> parasites are defective in metacyclogenesis. WT, *spt2*<sup>-</sup>, *spt2*<sup>-</sup>/+SPT2 or *spt2*<sup>-</sup>/+SPT2\* cells were grown to stationary phase (day 3) and the percentage of metacyclics was determined by the peanut agglutinin method.



**Fig. 3.** *Leishmania major* WT (grown in the absence or presence of 10  $\mu$ M myriocin), *spt2*<sup>-</sup> and *spt2*<sup>-</sup>/*SPT2* cells were cultured to mid-log phase ( $\sim 5 \times 10^6$  cells/ml) and pulse labeled with [<sup>3</sup>H]serine as described in Materials and methods. Cellular lipids were extracted and resolved by TLC. Each lane contained material from  $3 \times 10^7$  cells. Abbreviations: IPC, inositolphosphorylceramide; PE, phosphatidylethanolamine; PS, phosphatidylserine.

expression was restored (Figure 2C), or when *spt2*<sup>-</sup> was grown in the presence of SBs (below). Unsurprisingly, stationary phase *spt2*<sup>-</sup> cells showed poor infectivity in macrophage and animal infections (data not shown).

#### ***spt2*<sup>-</sup> lacks de novo synthesis of IPCs and ceramide**

Log phase parasites were pulse labeled with [<sup>3</sup>H]serine, and cellular lipids were extracted and separated by thin layer chromatography (Figure 3). *Leishmania major* SLs occur as ceramides and IPCs (Denny *et al.*, 2001; Ralton *et al.*, 2002), which were distinguished from other phospholipids arising through metabolism of serine such as phosphatidylserine (PS) or phosphatidylethanolamine (PE) by their mobility and resistance to mild base treatment. In WT abundant labeling of ceramides and IPCs was seen, which was absent in *spt2*<sup>-</sup>, (Figure 3). Ceramide and IPC synthesis was restored in the *spt2*<sup>-</sup> parasites after re-expression of *SPT2* (Figure 3, *spt2*<sup>-</sup>/*SPT2*). Similarly, treatment of WT with 10  $\mu$ M myriocin, a specific inhibitor of SPT (Mandala and Harris, 2000) did not limit growth, but inhibited synthesis of both IPCs and ceramides (Figure 3, WT + Myr).

#### ***spt2*<sup>-</sup> lacks sphingolipids**

We used electrospray ionization mass spectrometry (ESI/MS, negative ion mode) to examine SLs in total lipid fractions. The molecular species represented by [M-H]<sup>-</sup> or [M+Cl]<sup>-</sup> ions were determined by collisionally activated

dissociation (CAD) of the parent ion and mass analyses of the resultant product ions as described in Materials and methods.

In log phase WT parasites, a variety of IPCs and alkyl/acyl and acyl/acyl glycerophospholipids were identified by their [M-H]<sup>-</sup> ions (Figure 4). Glycerophospholipids identified included 1-alkyl (18:0)-2-acyl (18:1)-PI (1-*O*-octadecanoyl-2-octadecenoyl-*sn*-glycero-3-phosphoinositol, represented by [M-H]<sup>-</sup> at *m/z* 849.7, Figure 4A), which is probably a precursor in GPI anchor biosynthesis (McConville and Bacic, 1989; Ilgoutz and McConville, 2001), and diacyl (18:0/18:1)-PI (1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoinositol, represented by [M-H]<sup>-</sup> at *m/z* 863.7, Figure 4A). IPC species were represented by ions at *m/z* 778.6 and 806.7 corresponding to IPC-d16:1 (phosphoryl inositol *N*-stearoylhexadecespHING-4-ene, d16:1/18:0-PI-Cer) and IPC-d18:1 (phosphoryl inositol *N*-stearoylsphingosine, d18:1/18:0-PI-Cer), respectively, with IPC-d16:1 being more abundant (Figure 4A) (Kaneshiro *et al.*, 1986). Correspondingly, in WT parasites the ceramide precursor of IPC-d16:1 (d16:1/18:0-PI-Cer) was represented by low abundance [M-H]<sup>-</sup> and [M+Cl]<sup>-</sup> ions at *m/z* 536.6 and 572.5, respectively (Figure 4A inset). Thus, *Leishmania* preferentially incorporate myristoyl-CoA over palmitoyl-CoA into IPCs. In contrast, enzymic studies of yeast SPT showed that while palmitoyl Co-A was the most active, C12-C18 acyl-CoAs could be incorporated efficiently (Pinto *et al.*, 1992b) and *in vivo* SBs arise primarily from C16 and C18 acyl-CoA (Dickson *et al.*, 1997).

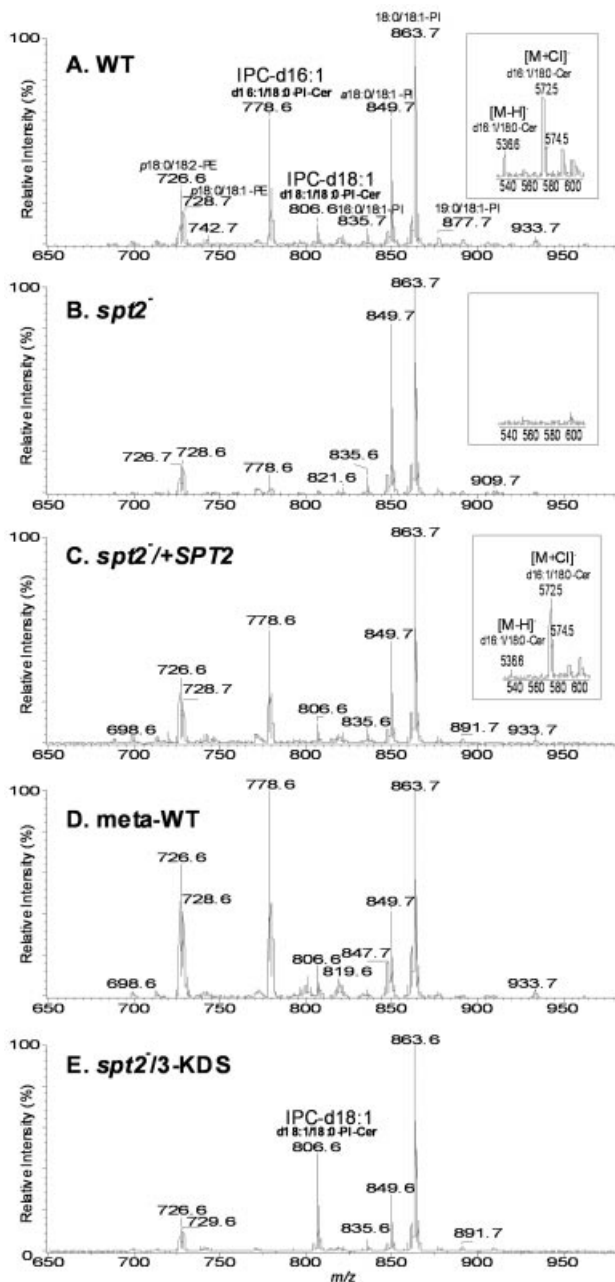
While *spt2*<sup>-</sup> lacked IPCs, as well as ceramide, it maintained WT levels of the other alkyl/acyl and acyl/acyl phospholipids (Figure 4B and inset). Restoration of *SPT2* expression in the *spt2*<sup>-</sup>/*SPT2* line restored IPCs and ceramide expression to the levels and patterns seen in WT (Figure 4C and inset). Thus deletion of *SPT2* abolished synthesis of SLs without affecting other phospholipids.

Total lipids from stationary phase or metacyclic WT parasites showed a similar profile of lipid species to that in log phase (metacyclic data is in Figure 4D; stationary phase data are not shown), although the relative abundance of IPCs and PEs increased in metacyclics (Figure 4D). Stationary phase *spt2*<sup>-</sup> resembled log phase in lacking IPCs and ceramides (data not shown); it was not possible to examine *spt2*<sup>-</sup> metacyclics due to their absence.

#### **Rescue of *spt2*<sup>-</sup> parasites by exogenous SBs but not ceramides**

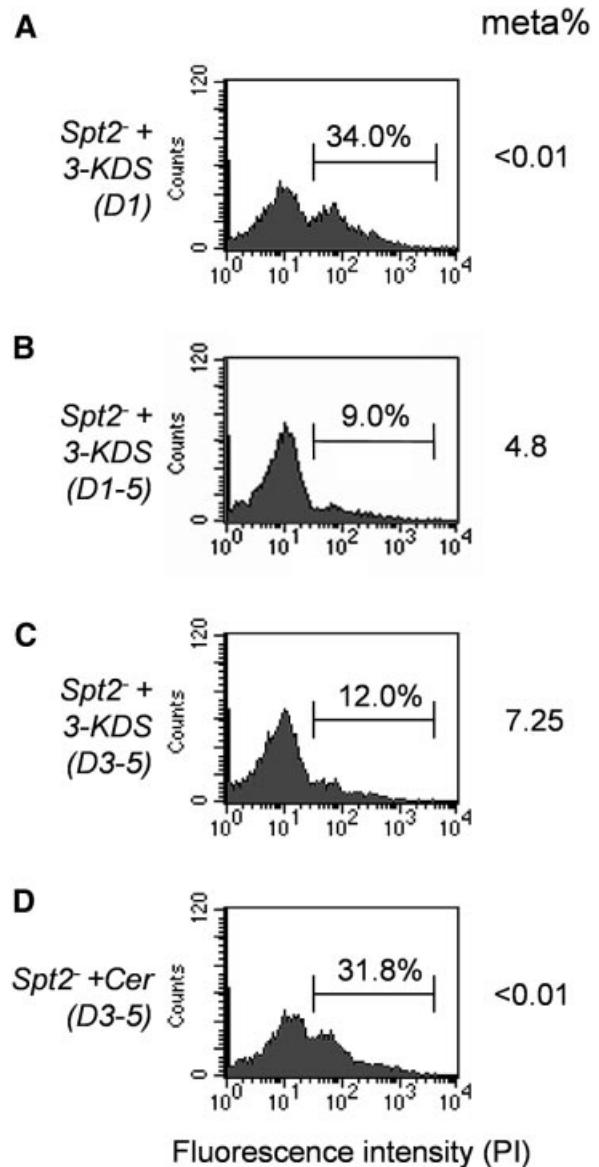
Provision of *spt2*<sup>-</sup> with the SPT product 3-keto-dihydro-sphingosine (3-KDS) or other SBs at 2–10  $\mu$ M, did not rescue *spt2*<sup>-</sup> (Figure 5A and data not shown; as in other organisms, SBs are toxic for *L. major* at higher concentrations). We found that the stability of SBs in *Leishmania* culture media was poor, and in other eukaryotes SLs are strictly regulated through both synthetic and degradative pathways (Hannun and Obeid, 2002; van Meer and Lisman, 2002). This prompted tests using more constant levels of SB supplementation, and daily addition of 3-KDS to 2  $\mu$ M completely rescued the *spt2*<sup>-</sup> defects (Figure 5B). IPC synthesis was restored in these cells, although only IPC-d18:1 was made as expected (Figure 4E).

Rescue of the stationary phase defect was also obtained with dihydrosphingosine, sphingosine and phytosphingo-



**Fig. 4.** Negative-ion ESI/MS spectra of total lipids purified from log phase WT (A), *spt2*<sup>-</sup> (B), *spt2*<sup>-</sup>/SPT2 (C), metacyclic WT (D) and *spt2*<sup>-</sup> cells grown with daily supplementation of 3-KDS (E). The assigned identities of peaks are indicated. Abbreviations: *p*18:0/18:2-PE, 1-*O*-octadec-1'-enyl-2-octadecadienoyl-*sn*-glycero-3-phosphoethanolamine (plasmalogen PE); *p*18:0/18:1-PE, 1-*O*-octadec-1'-enyl 2-octadecanoyl *sn*-glycero-phosphoethanolamine; d16:1/18:0-PI-Cer, phosphoryl inositol *N*-stearoylhexadecaphing-4-enine (IPC-d16:1); d18:1/18:0-PI-Cer, phosphoryl inositol *N*-stearoylsphingosine (IPC-d18:1); 18:0/18:1-PI, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoinositol; 16:0/18:1-PI, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoinositol; *a*18:0/18:1-PI, 1-*O*-octadecanoyl-2-octadecanoyl-*sn*-glycero-3-phosphoinositol (plasmalogen phosphoinositol). The insets in (A), (B) and (C) show the presence of *N*-stearoylhexadecaphing-4-enine (d16:1/18:0-Cer; [M-H]<sup>-</sup> and [M-C]<sup>-</sup> represent deprotonated and chloride ion addition of d16:1/18:0-Cer) in WT and *spt2*<sup>-</sup>/SPT2 but not in *spt2*<sup>-</sup> parasites.

sine (PHY); as before, ESI-MS spectrometry showed that only IPC-d18:1 synthesis was restored (data not shown). Supplementation with PHY resulted in the formation of a



**Fig. 5.** *spt2*<sup>-</sup> parasites can be rescued by SBs but not ceramides. (A) *spt2*<sup>-</sup> parasites were inoculated at  $2 \times 10^5$  cells/ml in M199/10% FBS medium containing 2  $\mu$ M of 3-KDS. (B) *spt2*<sup>-</sup> parasites were inoculated at  $2 \times 10^5$  cells/ml in M199/10% FBS medium and 2  $\mu$ M of 3-KDS was added each day from day 1 to day 5. (C) *spt2*<sup>-</sup> parasites were inoculated at  $2 \times 10^5$  cells/ml in M199/10% FBS medium and 2  $\mu$ M of 3-KDS was added each day from day 3 to day 5. (D) Similar experiments to (C) but 2  $\mu$ M of C18-ceramide was added instead of 3-KDS. At day 6 (approximately day 3 in stationary phase), the percentage of PI-positive cells and metacyclics were determined as described.

new IPC with PHY as the long chain base (data not shown), which was not abundant in WT. Neither C16-ceramide (*N*-palmitoylsphingosine), C18-ceramide (*N*-stearoylsphingosine) nor sphingomyelin was able to rescue the stationary phase defects or restore IPC synthesis (Table I; Figure 5D; data not shown), similar to yeast and mammalian SPT-deficient mutants (Hanada *et al.*, 1992; Pinto *et al.*, 1992a).

The SL/SB rescue studies addressed a potential concern that while *spt2*<sup>-</sup> completely lacked SLs, the serum required to cultivate *Leishmania in vitro* contains submicromolar concentrations of complex SLs such as gangliosides (Chu *et al.*, 2000). This can now be understood since (i)

**Table I.** Rescue of *spt2*<sup>-</sup> by SBs but not by ceramide

Expt	Addition	Log day			Stationary day			% PI	% metacyclic	% IPC
		1	2	3	4	5	6			
1	SB	-	-	-	-	-	-	40	<0.01	0
2	SB	+	+	+	+	+	+	9	4.8	59
3	SB	+	-	-	-	-	-	34	<0.01	11
4	SB	+	+	-	-	-	-	33	<0.01	15
5	SB	+	+	+	-	-	-	23	0.1	27
6	SB	-	-	-	+	-	-	34	0.12	18
7	SB	-	-	-	+	+	-	14	3.2	45
8	SB	-	-	-	+	+	+	12	5.0	49
9	Cer	+	+	+	+	+	+	37	<0.01	0

*spt2*<sup>-</sup> parasites were inoculated at  $1 \times 10^5$ /ml at day 1. SB (3-KDS) or CER (C18 ceramide) was added on the days indicated by '+' to a final concentration of 2  $\mu$ M. Cultures entered stationary phase on day 4, and metacyclic formation (peanut agglutinin method) and IPC levels (ESI/MS) were evaluated on day 7. The relative abundance of IPCs (IPC-d18:1 + IPC-d16:1 for WT or IPC-d18:1 for SB-supplemented *spt2*<sup>-</sup>) was estimated by comparison with 18:0/18:1-PI whose abundance appeared constant, and normalized to the ratio seen in WT.

mammalian SLs such as sphingomyelins, or ceramides derived from complex SLs, were unable to rescue *spt2*<sup>-</sup> defects; (ii) both *Leishmania* cells and culture media rapidly degraded SBs; and (iii) even active SBs must be added frequently at elevated levels. For these reasons, the *spt2*<sup>-</sup> parasite remained completely devoid of SLs.

#### **Spingoid bases are required in stationary phase but not in log phase**

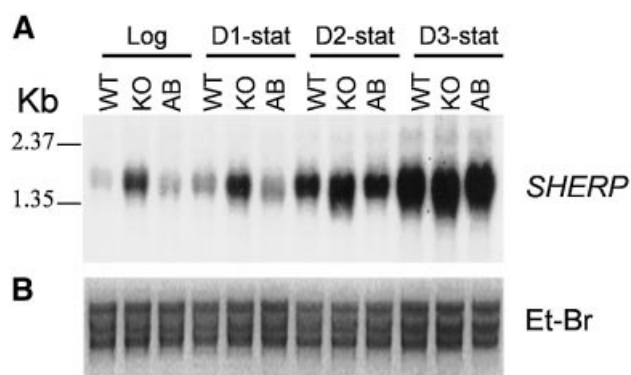
Rescue of the *spt2*<sup>-</sup> phenotypes with exogenous SBs permitted a test of the timing of their requirement during parasite growth. Although *SPT2* mRNA and protein levels were highest in log phase, provision of SBs only during this period failed to rescue the stationary phase *spt2*<sup>-</sup> defects (Table I). However, if SBs were added at the first day of stationary phase and maintained for at least 2 days, rescue was strong and similar to that obtained in parasites to which SBs had been added every day (Table I; Figure 5C). Rescue of viability and metacyclogenesis correlated with IPC synthesis, with an apparent threshold of ~30% of WT levels (Table I).

#### ***spt2*<sup>-</sup> mutants continue to express the metacyclic gene marker *SHERP***

To test whether expression of metacyclic stage genes was generally altered in *spt2*<sup>-</sup> due to disruption of SL-dependent signaling, we examined the expression of the metacyclic stage-specific marker *SHERP* (Knuepfer *et al.*, 2001). In log phase WT, *spt2*<sup>-</sup> and *spt2*<sup>-</sup>/*+SPT2*, *SHERP* mRNA levels were low, while upon entry into stationary phase *SHERP* transcripts increased dramatically (Figure 6). A small increase in *SHERP* mRNA was seen in log phase and early stationary phase *spt2*<sup>-</sup> (Figure 6B), the significance of which is unknown. Thus, despite the failure to form metacyclics, *spt2*<sup>-</sup> parasites nonetheless expressed a key metacyclic marker in stationary phase.

#### ***spt2*<sup>-</sup> defects do not arise through programmed cell death**

One explanation for the stationary phase *spt2*<sup>-</sup> defects invoked apoptosis or programmed cell death, since in other organisms SLs have both pro- and anti-apoptotic effects, and that at certain times, metazoan cells must choose between paths leading to either differentiation or death via apoptosis (reviewed by Vaux and Korsmeyer,

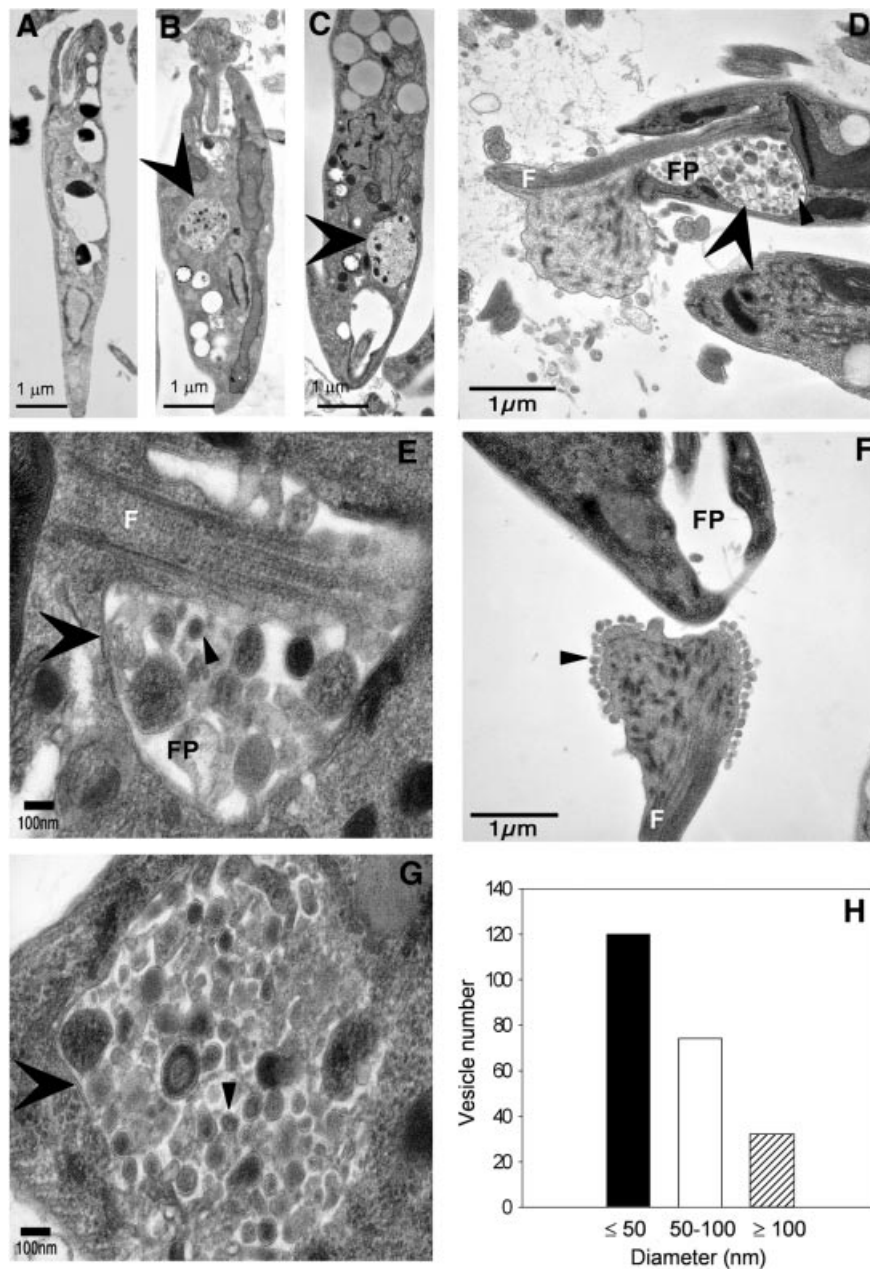


**Fig. 6.** Deletion of *SPT2* does not alter the expression of metacyclic stage-specific marker *SHERP*. (A) Northern blotting with radio-labeled *SHERP* probe was performed as described. Total RNA from log phase and day 1–3 stationary cultures was analyzed. (B) Loading control (ribosomal RNA stained with ethidium bromide). KO: *spt2*<sup>-</sup>, AB: *spt2*<sup>-</sup>/*+SPT2*.

1999; Maceyka *et al.*, 2002). Effectively, in stationary phase WT *Leishmania* chooses 'differentiation' while *spt2*<sup>-</sup> mutants choose 'death'. We examined several key parameters described previously for cell death in *Leishmania*: nuclear morphology, DNA fragmentation, surface exposure of PS and altered membrane permeability (Lee *et al.*, 2002; Zangger *et al.*, 2002), and focused on the critical times surrounding entry into stationary phase. In no respect did stationary phase *spt2*<sup>-</sup> parasites show characteristics of programmed cell death (Supplementary data).

#### ***spt2*<sup>-</sup> shows severe defects in membranous structures and small vesicle accumulation upon entry into stationary phase**

Transmission electron microscopy (EM) of stationary phase *spt2*<sup>-</sup> parasites revealed one or more of the following abnormalities: (i) increased vacuolization (Figure 7B and C, versus A for WT); (ii) extensive debris in the flagellar pocket (Figure 7D and E); (iii) alterations in the flagellar surface (Figure 7F), and (iv) accumulation of small vesicles with single lipid bilayer membranes in both internal vacuoles, the flagellar pocket and spilling out onto the parasite surface and flagellar membrane (Figure 7D–



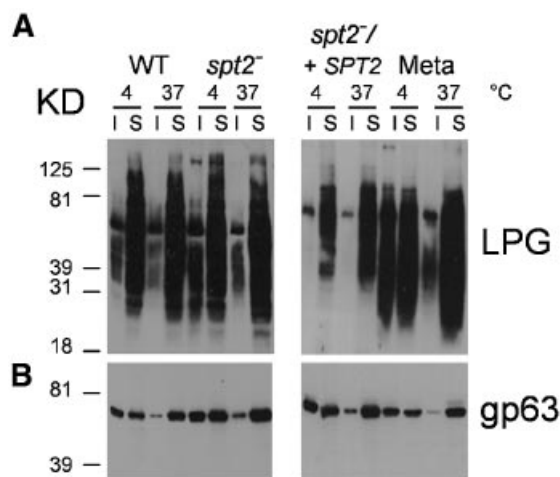
**Fig. 7.** *spt2*<sup>-</sup> parasites have severe defects in vesicular trafficking in stationary phase. (A)–(C) Transmission EM images of stationary phase WT and *spt2*<sup>-</sup> cells (day 3). Big arrowheads indicate vacuoles filled with small vesicles seen in *spt2*<sup>-</sup> (B and C) but not in WT (A) cells. (D)–(G) High magnification images of stationary phase *spt2*<sup>-</sup> showing MVB-like vesicles accumulated in and outside the flagellar pocket (D and E), at the surface of flagellum near the proximal end (F) and in an intracellular vacuole (G). Big arrowheads indicate flagellar pockets (D and E) or intracellular vacuoles (B, C and G). Small arrowheads indicate examples of MVB-like vesicles. F, flagellum; FP, flagellar pocket. (H) Size distribution of vesicles found in (D)–(G).

G). The sizes of the small vesicles in these compartments were heterogeneous, with the majority at ~50 nm (Figure 7H). The small vesicles resembled those occurring normally within other organelles of the endocytic pathway of *Leishmania* such as MVBs or the lysosome-like MVT (Weise *et al.*, 2000; Ghedin *et al.*, 2001; Mullin *et al.*, 2001). Virtually every stationary phase *spt2*<sup>-</sup> cell showed these defects, albeit to varying extents and proportions, and a variety of more severe abnormalities were found. No such defects were seen in log phase *spt2*<sup>-</sup>, or stationary phase WT or *spt2*<sup>-</sup>/*SPT2* parasites (data not shown). In other organisms SLs are enriched at various points in the

endocytic pathway as well as in MVB-derived vesicles (David *et al.*, 1998; Kasahara and Sanai, 1999; McMaster, 2001; van Meer and Lisman, 2002; Wubbolts *et al.*, 2003), and the phenotypes seen in *spt2*<sup>-</sup> were consistent with a defect in these processes.

#### ***spt2*<sup>-</sup> maintains the ability to generate DRMs similar to those in WT**

SLs participate in the formation of membrane microdomains often termed lipid ‘rafts’ (Simons and Ikonen, 1997; Simons and Toomre, 2000; van Meer and Lisman, 2002). One common method for their recovery is the



**Fig. 8.** *spt2*<sup>-</sup> parasites maintain TX-100 DRMs. Soluble (S) and insoluble (I) fractions from log and metacyclic phase WT *L. major*, and from log phase *spt2*<sup>-</sup> and *spt2*<sup>-/+SPT2</sup> parasites were subjected to western blot analysis to assess the distribution of LPG (A) and gp63 (B). Extractions were performed at 4°C or 37°C.

formation of detergent insoluble complexes at low but not high temperatures (DRMs) (Brown and London, 1998; London and Brown, 2000; Schuck *et al.*, 2003), and this has been used successfully in *Leishmania* (Denny *et al.*, 2001; Ralton *et al.*, 2002; Zufferey *et al.*, 2003). Thus, we prepared cold Triton X (TX)-100 DRMs from log and metacyclic phase WT *L. major*, and from log phase *spt2*<sup>-</sup> parasites, and probed them for the distribution of informative GPI-anchored markers.

In log and metacyclic WT parasites, gp63 showed a strong association with DRM fractions, as ~50% was insoluble to TX-100 extraction at 4°C but not at 37°C (Figure 8B). Gp63 in log phase *spt2*<sup>-</sup> parasites behaved identically to that in WT (Figure 8B). Similar results were obtained with other parasite DRM markers tested, and by additional criteria such as flotation in density gradients (data not shown). This suggested that the propensity of the *spt2*<sup>-</sup> mutant to form DRMs was unaltered, despite the absence of SLs (Figure 8).

Previously it was proposed that in contrast to other markers, LPG was associated with buoyant DRM fractions in metacyclic, but not log phase promastigotes (Denny *et al.*, 2002; Zufferey *et al.*, 2003). This finding was reproduced here with log phase WT or *spt2*<sup>-</sup>, as LPG was soluble at both 4°C and 37°C (Figure 8A), while a significant proportion of LPG now appeared in the DRM fraction in metacyclic WT parasites (Figure 8A). This conclusion was confirmed by flotation in density gradients (data not shown). It was not possible to examine *spt2*<sup>-</sup> metacyclics. Since the structure of the LPG GPI anchor is invariant during development (McConville *et al.*, 1992), relocalization of LPG to DRMs in metacyclic *Leishmania* may reflect a stage-specific reorganization of parasite membrane domains.

## Discussion

In this study, we probed the role of *de novo* SL synthesis in *Leishmania* growth and differentiation by studying a null mutant lacking *SPT2*, one of the subunits of the first

enzyme of the *de novo* SL pathway. The *SPT2* protein contains the SPT catalytic site (reviewed by Hanada, 2003), and we showed that mutation of this site abolished activity in *Leishmania*. Unlike yeast and mammalian cells, *SPT2* was not essential for *L. major* viability or growth during log phase in the absence of SBs. This raised the possibility that the *spt2*<sup>-</sup> parasites were completely lacking in SLs. We showed that the *spt2*<sup>-</sup> mutant lacked *de novo* SL synthesis (Figure 3), and that parasites grown *in vitro* lacked SLs including the abundant IPCs, as well as precursors such as ceramide detectable by MS analysis (Figure 4). Thus, we conclude that *spt2*<sup>-</sup> is completely devoid of SLs, and does not require them for near-normal growth in log phase.

In contrast, upon entry into stationary phase *spt2*<sup>-</sup> parasites rapidly lost viability and failed to generate the infective metacyclic stage. That these defects arose solely through loss of SL synthesis was established by first, that *spt2*<sup>-</sup> parasites were not rescued by expression of an active-site mutant of *SPT2*<sup>\*</sup>, and secondly, that *spt2*<sup>-</sup> parasites could be rescued by exogenous SBs. Since *SPT2* mRNA and protein was detected in early stationary phase but not in metacyclic promastigotes (Figure 1), presumably SLs are required in stationary phase for the completion of metacyclogenesis, but not by metacyclic parasites themselves.

To explain the phenotypes of the *spt2*<sup>-</sup> mutant, we considered effects related to overall membrane composition (bulk), the formation of membrane microdomains or 'rafts', membrane trafficking and cell signaling. In terms of 'bulk', apparently *Leishmania* can compensate for the loss of the 5–10% of the cellular lipids comprised by SLs by increasing overall lipid synthesis generally, as no specific changes in membrane composition were detected in the *spt2*<sup>-</sup> line in this work (other than loss of SLs; Figure 4). A similar conclusion was reached in studies of another mutant lacking the ~10% of the cellular lipids comprised of ether phospholipids (Zufferey *et al.*, 2003).

SLs, through interactions involving their head groups, the SB and the hydrophobic anchor, associate with sterols and other molecules to form distinct membrane microdomains ('lipid rafts') within eukaryotic membranes (Simons and Ikonen, 1997; van Meer and Lisman, 2002). Here we used a standard experimental criterion for 'lipid rafts' involving the formation of cold TX-100 DRMs. In *Leishmania* as in other organisms, DRMs are rich in SLs, GPI-anchored proteins and sterols (Denny *et al.*, 2001; Ralton *et al.*, 2002). However, the GPI-anchored protein gp63 was localized to a similar extent in DRMs in both the WT and *spt2*<sup>-</sup> parasites (Figure 8). Moreover, preliminary data examining other markers and/or methods for raft preparation similarly showed retention of DRMs in *spt2*<sup>-</sup> as well (unpublished data). Notably, retention of DRMs in the *spt2*<sup>-</sup> mutant does not imply that they have exactly the same properties as WT. Studies in other eukaryotes have suggested that the properties of DRMs/rafts may differ among cell types and even within different compartments within the same cell (Simons and Toomre, 2000), as discussed further below.

The remarkable ability of *Leishmania* to maintain DRMs without SLs may arise from two unique properties of parasite membranes. First, instead of cholesterol,



*Leishmania* membranes contain ergosterol, which shows an increased propensity to form the underlying biophysical characteristics of rafts in studies of model membranes (Xu *et al.*, 2001). Secondly, ether lipids comprise ~10% of *Leishmania* phospholipids, in the form of alkyl/acyl PE, PI, PC and GPI-anchors of proteins and phosphoglycans (Beach *et al.*, 1979; Wassef *et al.*, 1985; Singh *et al.*, 1988; Zufferey *et al.*, 2003). Potentially, LPG and/or other abundant GPI-anchored molecules or ether lipids may compensate for the lack of SLs. While less extensively studied, ether lipids associate preferentially with lipid rafts, and studies of model membranes suggest they may also promote the formation of raft-like domains (Mattjus and Slotte, 1996; Ohvo-Rekila *et al.*, 2002; Pike *et al.*, 2002). Thus, the *Leishmania* membrane may provide a permissive environment for alterations in lipid composition, and maintenance of membrane microdomains or 'rafts' (Zufferey *et al.*, 2003).

We eliminated several models for the stationary phase defect of *spt2*<sup>-</sup>, involving SL-dependent alterations in the metacyclic gene expression program or the induction of cell death through apoptotic mechanisms (Figure 6; Supplementary data). Instead, stationary phase *spt2*<sup>-</sup> parasites suffered catastrophic membranous defects that ultimately led to their demise. These defects included the formation of large vacuoles containing membranous material and smaller vesicles, general alterations in vacuolar appearance and abundance, the presence of membranous material and small vesicles in the flagellar pocket, some of which seemingly can escape and bind to the cell surface, and alterations in the flagellar surface (Figure 7). While a variety of aberrant larger vesicles were seen, the most abundant class had a size of ~50 nm, similar to that seen within organelles of the *Leishmania* endocytic pathway such as the MVT or MVB, or in yeast or other organelles in the MVB pathway (Weise *et al.*, 2000; Ghedin *et al.*, 2001; Mullin *et al.*, 2001; Katzmann *et al.*, 2002; Waller and McConville, 2002; Wubbolts *et al.*, 2003). In mammalian cells, recent data suggest that while the membranes of late endosomes and the MVB are low in SLs and cholesterol and enriched for other lipids such as *lyso*-bis-phosphatidic acid, the vesicles residing within the MVBs have a complementary composition enriched in SLs and cholesterol (Gruenberg, 2001; Kobayashi *et al.*, 2002; Mobius *et al.*, 2003; Wubbolts *et al.*, 2003). Thus it is reasonable to propose that the accumulation of MVB-like vesicles in stationary phase *spt2*<sup>-</sup> arises from the lack of SLs. This further suggests that while not essential for the formation of MVB vesicles in *Leishmania*, SLs are required at subsequent steps in trafficking and/or degradation.

Current data suggest that stage-specific changes in the organization of the parasite membrane, as well as increased demands on membrane trafficking, contribute to the stationary phase specificity of the *spt2*<sup>-</sup> defects. In both *Leishmania mexicana* and *Leishmania donovani*, the activity of degradative pathways associated with the MVT/lysosomal compartments are elevated in stationary phase, a time when parasites are undergoing considerable remodeling while differentiating into the infective metacyclic form (Mullin *et al.*, 2001; Waller and McConville, 2002). MVB vesicles are known to play key roles in degradative processes, through both ubiquitin-dependent

and independent targeting routes (Gruenberg, 2001; Katzmann *et al.*, 2002). That *Leishmania* membranes undergo stage-specific reorganization was shown by the association of LPG with DRMs in metacyclic but not growing cells, whereas the GPI-anchored protein gp63 was always associated with DRMs (Figure 8; McConville *et al.*, 1992; Denny *et al.*, 2002; Zufferey *et al.*, 2003). Alterations in DRM properties have been associated with defects in membrane trafficking (van Meer and Lisman, 2002), and mammalian MVB-derived vesicles are rich in SLs and cholesterol and show raft-like properties in detergent extractions (Mobius *et al.*, 2003; Wubbolts *et al.*, 2003). Significantly, the MVT network was suggested to include lipid raft-like domains in *L.donovani* (Ghedini *et al.*, 2001). In total, the coincident appearance of *spt2*<sup>-</sup> defects with developmental changes in membrane properties, and increased demands on membrane trafficking through the MVT/MVB pathway upon entry into stationary phase and differentiation, are consistent with the known roles and distribution of SLs.

The *spt2*<sup>-</sup> studies raise a number of important points that will be the subject of future studies. To definitively explore the role of SLs in *Leishmania* membrane formation and trafficking, it will be necessary to develop a repertoire of markers for both DRM domains, and for MVBs and MVTs. Notably, while the *spt2*<sup>-</sup> cells are attenuated in infections of macrophages and mice, after some delay, mice infected with high numbers of *spt2*<sup>-</sup> go on to develop lesions that progress rapidly (data not shown). Since amastigotes also lack *SPT2* mRNA and protein (Figure 1), this may imply that *L.major* amastigotes, like promastigotes, may not require SBs and possibly SLs. Alternatively, amastigote acquisition of host cell SLs has been described (reviewed in McConville *et al.*, 2002), and perhaps *Leishmania* satisfy amastigote SL requirements through salvage. Lastly, *Leishmania* offers a new perspective from which to explore sphingolipid metabolic pathways, in a setting where organisms are viable and permissive to perturbations not possible in other organisms (such as the study of DRMs lacking SLs). This feature could be adapted for use in cell-based assays exploring the function of SL metabolic genes expressed heterologously in *Leishmania*.

## Materials and methods

### ***Cloning and sequencing of L.major SPT2 and molecular constructs***

From the *L.major* Genome Database, we identified a 1.6 kb DNA sequence that was homologous to the *SPT2* genes from other species; this was amplified by PCR, inserted into the expression vector pXG (Ha *et al.*, 1996), and its sequence was confirmed. The K372A mutation in the catalytic site mutant *SPT2*\* was introduced by oligonucleotide directed mutagenesis. The *SPT2* ORF was inserted into the pXG-GFP+2/ vector (B2952; Zufferey *et al.*, 2003) to give a *GFP::SPT2* fusion protein. Bacterial expression of a His-tagged *SPT2* was achieved following insertion into the pET15b vector, and purified protein was used to generate antisera in rabbits. Specific details are included as Supplementary data.

### ***Leishmania culture and transfection***

Wild type *L.major* LV39 clone 5 (Rho/SU/59/P) cells were grown in M199 medium with supplements and 10% heat inactivated fetal bovine serum (FBS) at 26°C. Metacyclic parasites were isolated by PNA agglutination or density gradient methods (Sacks and Perkins, 1984; Späth and Beverley, 2001). Stocks of SBs and ceramides were made at 10

and 1 mM, respectively in ethanol, and stored at  $-20^{\circ}\text{C}$ . Transfections by electroporation were performed as described (Kapler *et al.*, 1990). The *SPT2* coding region of the first allele was replaced by a *HYG* marker and that of the second by a *PAC* marker. Restoration of *SPT2* expression to several of these *spt2*- mutants was achieved by transfection of pXG-SPT2. Specific details are included as Supplementary data.

#### Flow cytometry, fluorescence and electron microscopy

Cells were attached to slides, fixed with formaldehyde and permeabilized with ethanol. Antisera used included rabbit anti-*Trypanosoma brucei* BIP and monoclonal antibodies against gp63 and LPG (WIC 79.3). For electron microscopy, parasites were fixed in 2% formaldehyde/2.5% glutaraldehyde, washed, post-fixed with 1% osmium tetroxide and stained with 1% uranyl acetate; 70–80 nm sections were cut and viewed by transmission EM. For flow cytometry,  $1 \times 10^6$  cells were washed once in phosphate-buffered saline (PBS) and incubated with 0.5  $\mu\text{g}/\text{ml}$  of propidium iodide at room temperature for 5 min, 1  $\mu\text{g}/\text{ml}$  of annexin V-FITC (Sigma) on ice for 30 min or 10  $\mu\text{M}$  YOPRO-1 (Molecular Probes) at room temperature for 10 min. Flow cytometry was performed using a Becton Dickinson FACSCalibur system. Specific details are found in Supplementary data.

#### Metabolic labeling, lipid fractions and TLC

Log phase cells ( $<5 \times 10^9/\text{ml}$ ) were resuspended in M199/10% FCS at  $1 \times 10^8$  cells/ml and labeled with [ $^3\text{H}$ ]serine (26 Ci/mmol) at 50  $\mu\text{Ci}/\text{ml}$  for 20 min at room temperature. Cells were harvested, washed with PBS, and resuspended in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM  $\text{CaCl}_2$ , 0.5% NP-40, 0.5% TX-100, 1 mM PMSF, 2  $\mu\text{g}/\text{ml}$  leupeptin, 5  $\mu\text{g}/\text{ml}$  pepstatin A) at  $5 \times 10^7$  cells/ml. After 10 min on ice, lysates were extracted with 3.75 vol of  $\text{CHCl}_3/\text{methanol}$  (1:2), followed by 1.25 vol of  $\text{CHCl}_3$ , and 1.25 vol of water. After centrifugation (1000g, 10 min), the upper aqueous phase was removed and the organic phase was washed once with water. The organic phase was then dried under nitrogen and dissolved in  $\text{CHCl}_3$  at the equivalent of  $2 \times 10^9$  cells/ml and stored at  $-20^{\circ}\text{C}$ . TLC analysis of lipids was performed using aluminum-backed silica gel plates (Sigma). Solvent contained methyl acetate/1-propanol/ $\text{CHCl}_3/\text{methanol}/0.25\%$  KCl (25:25:25:10:9). Autoradiography was performed after spraying plates with  $\text{En}^3\text{Hancer}$  (Perkin Elmer). The presence of SBs in ceramides and IPCs was confirmed by resistance to mild base (0.1 M KOH in methanol,  $37^{\circ}\text{C}$ , 3 h).

#### Mass spectrometry

For MS, cells were washed in PBS and resuspended in water at  $2 \times 10^8$  cells/ml, sonicated on ice (15 W, 5 s) and extracted as described for TLC. ESI/MS analyses were performed in the negative ion mode, and ESI/MS or ESI/MS/MS spectra were generated by a 1 min or 1–20 min period, respectively, of signal averaging of repeated scans. Specific details of instrumentation and protocols can be found in Supplementary data.

#### Preparation of DRMs and western blotting of LPG and gp63

*Leishmania* cell pellets ( $1 \times 10^8$  cells) were washed once in PBS and extracted with 1 ml of 1% TX-100 (prepared in PBS and supplemented with 1 mM PMSF, 2  $\mu\text{g}/\text{ml}$  leupeptin and 5  $\mu\text{g}/\text{ml}$  pepstatin A) for 10 min on ice or at  $37^{\circ}\text{C}$ . Detergent-soluble and insoluble fractions were separated by centrifugation at 20 000g for 2 min at  $4^{\circ}\text{C}$  or  $37^{\circ}\text{C}$ . An equal volume of  $2 \times$  SDS sample buffer was added to the detergent soluble fraction (supernatant) and two volumes of  $1 \times$  SDS sample buffer were added to the detergent insoluble fraction (pellet). Samples were resolved by SDS-PAGE and electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Biosciences). LPG was detected with monoclonal antibody WIC79.3 (1:1000). Gp63 was detected with monoclonal antibody 235 (1:1000), (Connell *et al.*, 1993). An enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) was used to detect signals.

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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