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Sphingosine kinase A is a pleiotropic and essential enzyme for *Leishmania* survival and virulence

Ou Zhang,[1†] Fong-Fu Hsu,[2] Wei Xu,[1] Mattie Pawlowic[1] and Kai Zhang[1*]
[1]*Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409, USA.*
[2]*Department of Internal Medicine, Washington University School of Medicine, 660 S. Euclid Ave., Saint Louis, MO 63110, USA.*

Summary

Sphingosine kinase is a key enzyme in sphingolipid metabolism, catalysing the conversion of sphingosine or dihydrosphingosine into sphingosine-1-phosphate or dihydrosphingosine-1-phosphate respectively. In mammals, sphingosine-1-phosphate is a powerful signalling molecule regulating cell growth, differentiation, apoptosis and immunity. Functions of sphingosine kinase or sphingosine-1-phosphate in pathogenic protozoans are virtually unknown. While most organisms possess two closely related sphingosine kinases, only one sphingosine kinase homologue (SKa) can be identified in *Leishmania*, which are vector-borne protozoan parasites responsible for leishmaniasis. *Leishmania* SKa is a large, cytoplasmic enzyme capable of phosphorylating both sphingosine and dihydrosphingosine. Remarkably, deletion of SKa leads to catastrophic defects in both the insect stage and mammalian stage of *Leishmania* parasites. Genetic and biochemical analyses demonstrate that proper expression of SKa is essential for *Leishmania* parasites to remove toxic metabolites, to survive stressful conditions, and to cause disease in mice. Therefore, SKa is a pleiotropic enzyme with vital roles throughout the life cycle of *Leishmania*. The essentiality of SKa and its apparent divergence from mammalian counterparts suggests that this enzyme can be selectively targeted to reduce *Leishmania* infection.

Accepted 24 August, 2013. *For correspondence. E-mail kai.zhang@ttu.edu; Tel. (+1) 806 742 2710 ext 250; Fax (+1) 806 742 2963. †Present address: Department of Microbiology and Immunology, University of Michigan Medical School, 1150 West Medical Center Drive, 5641 Medical Science II, Ann Arbor, MI 48109, USA.

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Introduction

Trypanosomatid parasites of the genus *Leishmania* cause a spectrum of serious diseases (collectively known as leishmaniasis) infecting 10–12 million people in 88 countries (Cunningham, 2002). During their life cycle, these vector-borne protozoans alternate between flagellated promastigotes which live in the midgut of sandflies and non-flagellated amastigotes which reside in the phagosome of mammalian macrophages. Control of leishmaniasis is hampered by the lack of a safe vaccine, limitations of frontline drugs, and the emergence of drug resistant strains (Santos *et al*., 2008). To develop better treatments, it is necessary to decipher the molecular strategy utilized by *Leishmania* parasites to thrive under the harsh conditions in sandflies and humans.

Sphingolipids (SLs) and their metabolic intermediates are well-characterized bioactive molecules in mammals with diverse roles in raft formation, signal transduction, cell-to-cell recognition, and immune responses (Merrill *et al*., 1993; Vaux and Korsmeyer, 1999; Kolter *et al*., 2002; Maceyka *et al*., 2002). Functions of SL metabolism in *Leishmania* have been probed using gene knockout mutants and their reconstituted strains (add-backs). Previously, a *spt2*⁻ mutant that lacks an essential subunit of serine palmitoyltransferase (SPT) was generated in *Leishmania major* through targeted gene deletion (Zhang *et al*., 2003; Denny *et al*., 2004). SPT is responsible for the *de novo* synthesis of sphingoid bases including 3-ketodihydrosphingosine and dihydrosphingosine (DHS) (Denny *et al*., 2004) (Zhang *et al*., 2003) (Fig. S1), which are intermediates for the production of high order SLs. Other sphingoid bases such as sphingosine (Sph) can be generated through the degradation of ceramide (Mao and Obeid, 2008) (Fig. S1). *Spt2*⁻ mutant is replicative in culture during the logarithmic phase but loses viability during the stationary phase and fails to differentiate from non-infective procyclics to infective metacyclics (a process known as metacyclogenesis in *Leishmania* (Sacks and Perkins, 1985)) (Zhang *et al*., 2003). Very similar phenotypes are also displayed by another *L. major* mutant named *spl*⁻ which lacks the enzyme sphingosine-1-phosphate lyase (SPL) (Zhang *et al*., 2007). SPL catalyses the hydrolysis of phosphorylated sphingoid bases such as sphingosine-1-phosphate (S1P) and

dihydrosphingosine-1-phosphate (DHS1P) (Fig. S1). Importantly, supplementation of ethanolamine (EtN) completely reversed the viability and differentiation defects of both *spt2*⁻ and *spl*⁻ promastigotes (Zhang *et al*., 2007). After being taken up by cells, EtN can be converted into EtN-phosphate (EtN-P, a downstream product of SPL; Fig. S1) and then further utilized to synthesize plasmenylethanolamine (PE), which is an abundant phospholipid in *Leishmania* (Zhang *et al*., 2003; 2007; Gibellini *et al*., 2008). Therefore, a main function of sphingoid base synthesis and degradation is to generate EtN-P, a metabolite essential for phospholipid synthesis and metacyclogenesis in *Leishmania* promastigotes (Fig. S1).

Recently, the turnover of high order SLs was explored in *Leishmania* parasites. In *L. major*, a single inositol phosphosphingolipid phospholipase C-like (ISCL) protein is responsible for the degradation of both inositol phosphorylceramide (IPC, the predominant SL in *Leishmania*) and sphingomyelin (an abundant SL in mammals) (Zhang *et al*., 2009). Null mutants of *ISCL* (*iscl*⁻) grew and differentiated normally as promastigotes but were hypersensitive to acidic pH (Xu *et al*., 2011). These mutants could not cause disease in either immunocompetent or immunocompromised mice, although they were able to persist at low levels at the site of infection (Xu *et al*., 2011). Further studies demonstrate that the ability to degrade IPC is important for acid tolerance in promastigotes, and the ability to degrade host-derived sphingomyelin is required for amastigote replication and disease development in mammals (Xu *et al*., 2011, O. Zhang *et al*., 2012). A parallel study was performed in another *Leishmania* species, *L. (L) amazonensis*, and the phenotype of *Laiscl*⁻ mutant mostly mimics that of *L. major iscl*⁻ except that the outcome of *Laiscl*⁻ infection is highly dependent on the genetic background of mice (Pillai *et al*., 2012). Together, these findings strongly suggest that degradation of high order SLs, especially host-derived sphingomyelin, plays a pivotal role in the proliferation of *Leishmania* in mammals and the manifestation of disease.

In addition to SPT, SPL and sphingomyelinase, another well-studied enzyme in SL metabolism is sphingosine kinase (SK) which converts Sph or DHS into S1P or DHS1P respectively (Maceyka *et al*., 2002). In organisms as diverse as mammals, fruit fly, *C. elegans* and yeasts, two SK isoforms (SK1 and SK2) are responsible for the phosphorylation of sphingoid bases using ATP as the phosphate donor (Maceyka *et al*., 2002). Mammalian SK1 and SK2 exhibit overlapping substrate specificity but are not redundant due to different developmental expression, tissue distribution and subcellular localization (Kohama *et al*., 1998; Liu *et al*., 2000). For example, SK1 is predominantly cytosolic and can be recruited to the plasma membrane upon stimulation (Pitson *et al*., 2005; Stahelin *et al*., 2005), while SK2 shuttles between the cytoplasm and nucleus (Igarashi *et al*., 2003; Hait *et al*., 2009). In mammals, S1P (the product of SK1/SK2) is a potent signalling lipid that regulates a plethora of important cellular processes including proliferation, apoptosis, calcium homeostasis, angiogenesis, platelet formation, cell migration and immune responses (Strub *et al*., 2010; Pitson, 2011; L. Zhang *et al*., 2012). S1P mainly exerts its function by interacting with a family of cell surface G protein-coupled receptors ('inside-out signalling') to activate various signalling pathways (Rosen *et al*., 2009; Strub *et al*., 2010). S1P can also act as a second messenger by regulating intracellular targets such as histone deacetylases, mitochondrial prohibitin 2, and tumour necrosis factor receptor-associated factor 2 (Xia *et al*., 2002; Hait *et al*., 2009; Alvarez *et al*., 2010; Strub *et al*., 2011). Due to S1P's role in promoting cell survival and proliferation, human SKs have been developed as therapeutic targets for the treatment of certain cancer and inflammatory disorders (Shida *et al*., 2008; Karliner, 2009; Edmonds *et al*., 2011). In *Saccharomyces cerevisiae*, genetic studies suggest that cytoplasmic S1P can suppress cell growth and increase heat tolerance at the same time, although the intracellular targets of S1P in fungi have yet to be determined (Lanterman and Saba, 1998; Kim *et al*., 2000; Birchwood *et al*., 2001).

In contrast to the wealth of knowledge on S1P signalling in mammals, functions of SK and S1P have not been explored in parasitic protozoans such as trypanosomatids and apicomplexans, which are evolutionarily divergent from animals and fungi. In this study, we investigated a SK homologue in *L. major* named SKa. Based on our previous findings, SKa should be involved in the conversion of serine to EtN-P (Fig. S1) (Zhang *et al*., 2007). Since S1P regulates numerous processes in mammals, it is tempting to speculate whether SK/S1P possess any other roles in addition to EtN-P production. Here our genetic and biochemical analyses demonstrate that *Leishmania* SKa is a critically important and pleiotropic enzyme responsible for the detoxification of sphingoid bases (during the replicative log phase) and the regulation of stress response (during the non-replicative stationary phase). In addition, the expression level of SKa needs to be carefully controlled to ensure parasite survival in mice. These findings add new knowledge to the role of SL metabolism in *Leishmania* and may open doors to new anti-protozoan treatments.

Results

Identification and genetic manipulation of SKa in L. major

A single candidate *SK* gene was identified in the *L. major* genome (Tritryp DB system ID: LmjF26.0710) and desig-

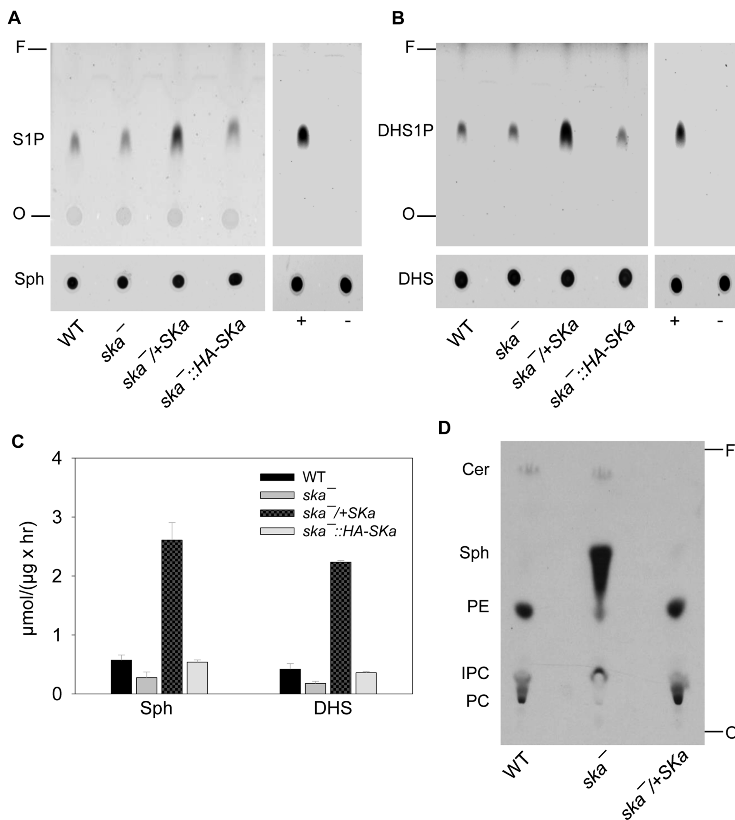

Fig. 1. *SKa* encodes a functional SK in *L. major*.
A–C. *In vitro* SK activity assay using *Leishmania* lysates. Promastigote lysates were incubated with NBD-Sph (A) or NBD-DHS (B) and lipid extraction was performed as described in *Experimental procedures*. Both the aqueous phase (top) and the organic phase were resolved by TLC (bottom). Positive control (+): 0.1 unit of purified human SK2; negative control (–): boiled WT lysate. These assays were repeated three times and results were quantified as μmol (μg × h)$^{-1}$ and summarized in C (error bars represent standard deviations).
D. Metabolism of serine in *L. major*. Early log phase promastigotes were labelled with [3-^{3}H]-serine for 48 h and total lipids were extracted and resolved by TLC as previously described (Zhang *et al.*, 2003). O: origin of migration; F: solvent front.

nated as *SKa*. Syntenic orthologues of *SKa* are conserved in other trypanosomatid pathogens including *Leishmania braziliensis*, *Leishmania infantum*, *Leishmania mexicana*, *Trypanosoma brucei* and *Trypanosoma cruzi*. While most organisms contain two SK isoenzymes, only one putative SK can be identified in *Leishmania* spp. and *Trypanosoma* spp. With 935 amino acids, the predicted *L. major* SKa protein is significantly larger than its orthologues in mammals and yeasts (Nagiec *et al.*, 1998; Liu *et al.*, 2002) (Fig. S2). It only shows 22–26% overall identity to human SK1/SK2 but does contain five conserved regions (C1-C5 in Fig. S2) and a diacylglycerol kinase domain (amino acid 343–421, underlined in Fig. S2B) which are evolutionarily conserved in all SKs (Pitson, 2011).

To examine the role of SKa, null mutants were generated in *L. major* through two rounds of targeted gene replacement as previously described (Kapler *et al.*, 1990) (Fig. S3A–C). Southern blot analyses with probes from the 5′-flanking region and the open reading frame confirmed the loss of *SKa* in two *ska⁻* clones (#2-1 and 2-3 in Fig. S3F–G). To complement these mutants, a high copy plasmid containing *SKa* (pXG–*SKa*) was introduced into *ska⁻* to generate an episomal add-back *ska⁻/ + SKa* (Fig. S3E and G). As expected, these *ska⁻/ + SKa* cells contained many more copies of *SKa* open read frame than WT parasites (Fig. S3G). To restore *SKa* expression to near-WT level, we inserted a HA-tagged *SKa* into the genomic locus of *SKa* to generate a chromosomal knockin add-back *ska⁻::HA-SKa* (*ΔSKa::BSD/ΔSKa::HA-SKa*), using a previously described approach for allelic replacement in *Leishmania* (Madeira da Silva *et al.*, 2009). Southern blot confirmed that these *ska⁻::HA-SKa* parasites had a single copy *SKa* at the correct locus (in Fig. S3F–G, the *SKa* open reading frame in *ska⁻::HA-SKa* is only detectable after 12 h of exposure).

SKa encodes a functional, cytoplasmic SK

To determine if *SKa* encodes a *bona fide* SK, we performed an *in vitro* activity assay by incubating promastigote lysates with 7-nitro-2-1,3-benzoxadiazol (NBD)-labelled Sph (Billich and Ettmayer, 2004). Following extraction, liquids from both the aqueous phase and organic phase were resolved by thin layer chromatography (TLC) (Fig. S4A and B). As expected, the majority of NBD-S1P was detected in the aqueous phase while the majority of NBD-Sph was retained in the organic phase. Compared with WT parasites, *ska⁻* mutants had a reduced yet still detectable level of SK activity (50~60% of WT-level) towards Sph and DHS (Fig. 1A–C). Notably, the chromosomal knockin add-back *ska⁻::HA-SKa* showed a similar SK level as WT parasites, whereas the episomal add-back *ska⁻/ + SKa* exhibited 3–5 time more activity

Table 1. Abundance of plasmenylethanolamine or PLE (×10^7 molecules per cell).

	WT	*ska*$^-$	*ska*$^-$/ + *SKa*
PLE (early-log)	7.2 ± 0.4	5.0 ± 0.5	6.5 ± 1.8
PLE (mid-log)	6.3 ± 3.0	4.6 ± 2.0	6.8 ± 4.6

Abundance of PLE in early log phase (1–2 × 10^6 cells ml^{-1}) or mid-log phase (4–6 × 10^6 cells ml^{-1}) promastigotes were determined by semi-quantative mass spectrometry. Average values from two experiments are summarized with standard deviations.

than WT (Fig. 1A–C), presumably due to increased *SKa* copy number (Fig. S3G). These data suggest that while *SKa* encodes an active enzyme which can phosphorylate both Sph and DHS, it may not be the only SK in *L. major*. In a metabolic labelling experiment, live WT and *ska*$^-$ promastigotes were incubated with [3-^{3}H]-serine for 48 h and then subjected to lipid analysis by TLC as previously described (Zhang *et al.*, 2007). As shown in Fig. 1D, WT parasites could metabolize serine into PE, PC, IPC and ceramide. In contrast, the incorporation of serine into PE and PC was much less robust in *ska*$^-$ promastigotes (Fig. 1D). Meanwhile, a significant accumulation of labelled sphingoid base was detected in *ska*$^-$ mutants and this was largely reversed in the *ska*$^-$/ + *SKa* parasites (Fig. 1D). Collectively, these data suggest that *L. major* possess more than one functional SK, although only SKa is recognizable in the genome.

To determine the impact of SKa deletion on lipid synthesis, we examined the composition of major phospholipids by mass spectrometry. As indicated in Fig. S5, the lipid profile (PE, PI, IPC and ceramide) was not drastically altered in log phase *ska*$^-$ parasites. This is likely due to the salvage of phospholipids and SLs from the culture medium which would bypass the sphingoid base metabolism (see the pathway in Fig. S1), similar to the log phase *spt2*$^-$ and *spl*$^-$ parasites (Zhang *et al.*, 2007). We also quantified the abundance of plasmenylethanolamine (PLE), which is the dominant type of PE in *Leishmania* (Zhang *et al.*, 2007). As summarized in Table 1, the level of PLE in log phase promastigotes of *ska*$^-$ was only slightly lower than that in WT or *ska*$^-$/ + *SKa* parasites (not statistically significant).

To determine where SKa is expressed in promastigotes (the sandfly stage), a GFP–SKa fusion protein was introduced into *L. major* WT parasites (on a high copy-number plasmid) and the transgenic WT/ + GFP–SKa cells displayed a high level of SK similar to that of *ska*$^-$/ + *SKa* (data not shown), indicating that the GFP-fusion protein was functional. By immunofluorescence microscopy, GFP–SKa was primarily found in the cytoplasm (Fig. 2A–D). Similar results were observed when the *ska*$^-$*::HA-SKa* promastigotes were probed with an anti-HA antibody (Fig. S6), indicating that the cytoplasmic localization is not an overexpression artefact. To examine the localization of SKa in amastigotes (the mammalian stage), murine macrophages infected with WT/ + GFP–SKa parasites were subjected to immunofluorescence analysis using an anti-GFP antibody. As illustrated in Fig. 2E–I, a clear cytosolic distribution was detected in intracellular amastigotes. Therefore, SKa is mainly expressed in the cytoplasm of *Leishmania* parasites.

Ska$^-$ promastigotes manifest severe growth defect due to sphingoid base accumulation

Immediately after obtaining the *ska*$^-$ knockout, we noticed that these mutants could only grow to 6–8 × 10^6 cells ml^{-1} in culture, which was only 1/4 to 1/3 of the maximal density for WT promastigotes (Fig. 3A). Afterwards, they gradually died in 3–4 days (Fig. 3A and B). When the viable, log phase *ska*$^-$ promastigotes (at 1–5 × 10^6 cells ml^{-1}) were inoculated in fresh media at 10^1–10^5 cells ml^{-1}, their growth rates and doubling times were nearly identical to those of WT parasites until culture densities reached 6–8 × 10^6 cells ml^{-1} (Fig. 3A and data not shown). Importantly, reconstitution of *SKa* expression through either chromosomal knockin (*ska*$^-$*::HA-SKa*) or episomal complementation (*ska*$^-$/ + *SKa*) fully restored *ska*$^-$ survival and proliferation in culture (Fig. 3A and B). Unlike the *spt2*$^-$ or *spl*$^-$ mutants (Zhang *et al.*, 2007), *ska*$^-$ parasites could not be rescued by exogenous EtN alone (Fig. 3C and D), indicating that SKa has additional roles beyond EtN-P production (Fig. S1). Exogenous S1P or DHS1P also failed to rescue *ska*$^-$ (Fig. S7), suggesting that the function of SKa is not mediated through membrane-bound receptors that recognize S1P or DHS1P. This is consistent with the lack of G-protein coupled receptors in *Leishmania* genome (Ivens *et al.*, 2005).

The rapid demise of *ska*$^-$ after reaching 6–8 × 10^6 cells ml^{-1} suggests that certain toxic metabolites are secreted by the mutant or released from dead parasites. Indeed, conditioned medium from dying *ska*$^-$ culture was deleterious to WT promastigotes (‘c’ in Fig. S8). In contrast, conditioned medium from healthy, low-density *ska*$^-$ culture could support parasite growth (‘a’ and ‘b’ in Fig. S8). Being the substrates of SK, sphingoid bases such as DHS and Sph are known to exert surfactant effect (as membrane disruptors) in mammalian cells (Contreras *et al.*, 2006). They are also potent inhibitors of protein kinase C (Hannun *et al.*, 1986; Merrill *et al.*, 1986). In culture, both DHS and Sph exhibited strong anti-proliferation activities (IC50 = 1–5 μM) against *L. major*, *L. amazonensis*, *L. mexicana*, and *L. donovani* (Fig. S9A–D and data not shown). By comparison, ceramides were not nearly as toxic (IC50 > 14 μM) (Fig. S9E

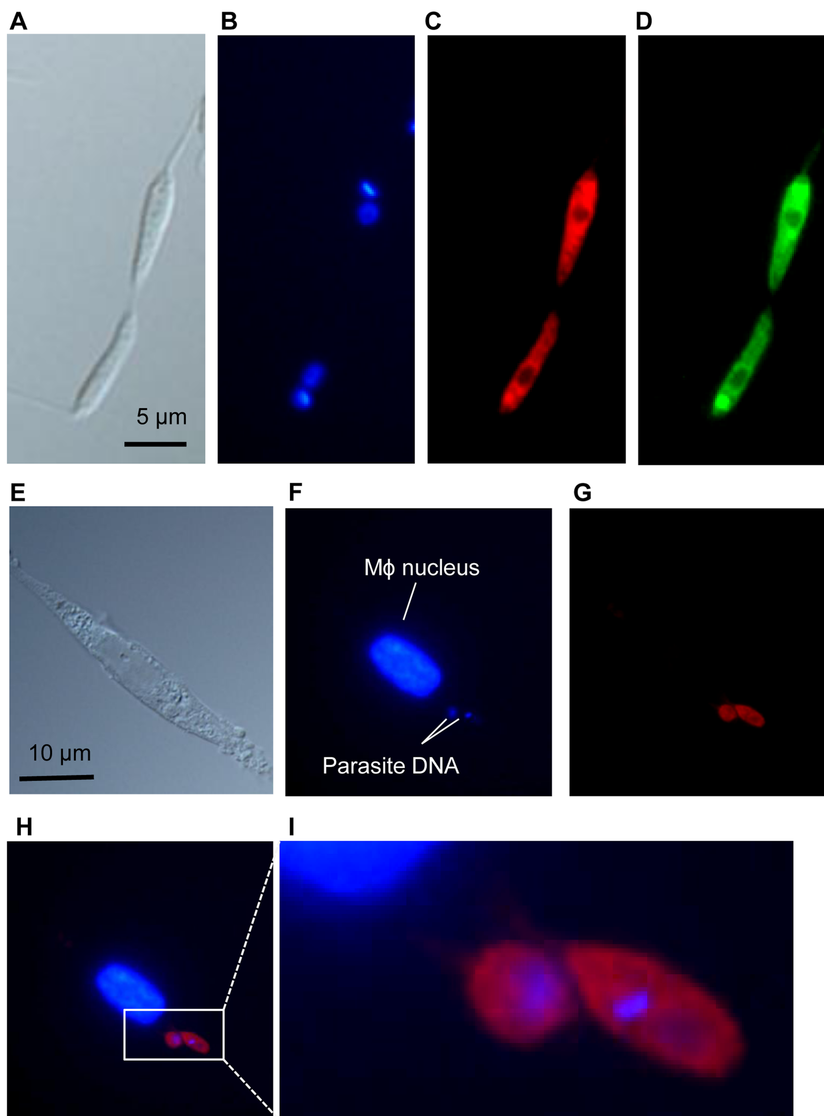

Fig. 2. Cytoplasmic localization of the SKa protein. WT/ + GFP–SKa promastigotes (A–D) or WT/ + GFP–SKa-infected MΦs (E–I) were permeabilized and subjected to immunofluorescence microscopy. (A and E) Differential interference contrast images; (B and F) DNA staining with Hoechst 33242; (C and G) rabbit anti-GFP polyclonal antibody labelling followed by incubation with a Texas red-conjugated goat anti-rabbit IgG antibody; (D) GFP fluorescence; (H) overlay of F and G; (I) enlarged image of the framed area in H.

and F). These findings, along with the lack of ceramide accumulation in *ska⁻* (Fig. 1D and Fig. S5), suggest that ceramides are unlikely to be the toxic metabolites.

To examine whether *ska⁻* died from sphingoid base accumulation, myriocin (MYR) was added to promastigote medium to block the *de novo* SL synthesis (Fig. S1) (Zhang *et al*., 2003). EtN was also provided to ensure EtN-P production (Fig. S1) (Zhang *et al*., 2007). Remarkably, the combination of MYR (1–10 μM) and EtN (100–500 μM) completely restored the viability, morphology, and proliferation rate of *ska⁻* (Fig. 3C and D; Fig. 4A and B) in culture. In addition, these chemically rescued mutants (*ska⁻* + MYR + EtN) produced similar amounts of metacyclics as WT, *ska⁻::HA-SKa* and *ska⁻/ + SKa* parasites in stationary phase (Fig. 4A). Without EtN, MYR alone partially rescued the growth and survival of *ska⁻* but not metacyclogenesis (Fig. 4A and B), which was similar to the *spt2⁻* mutant and in agreement with our previous finding that EtN-P production is required for differentiation (Zhang *et al*., 2007). Based on these findings, we conclude that a crucial role of SKa in *Leishmania* promastigotes is to avert the accumulation of toxic sphingoid bases (Fig. S1). The fact that *ska⁻* mutants still possess significant SK activity (Fig. 1) suggests that even a partial inhibition of SK can be detrimental to *Leishmania*. Unless otherwise specified, MYR (1 μM) and EtN (200 μM) were provided to *ska⁻* promastigote cultures in all the studies described below.

Ska⁻ promastigotes are extremely vulnerable to stress

To establish infection and proliferate in the mammalian host, *Leishmania* parasites must develop resistance to various stress conditions including elevated temperature,

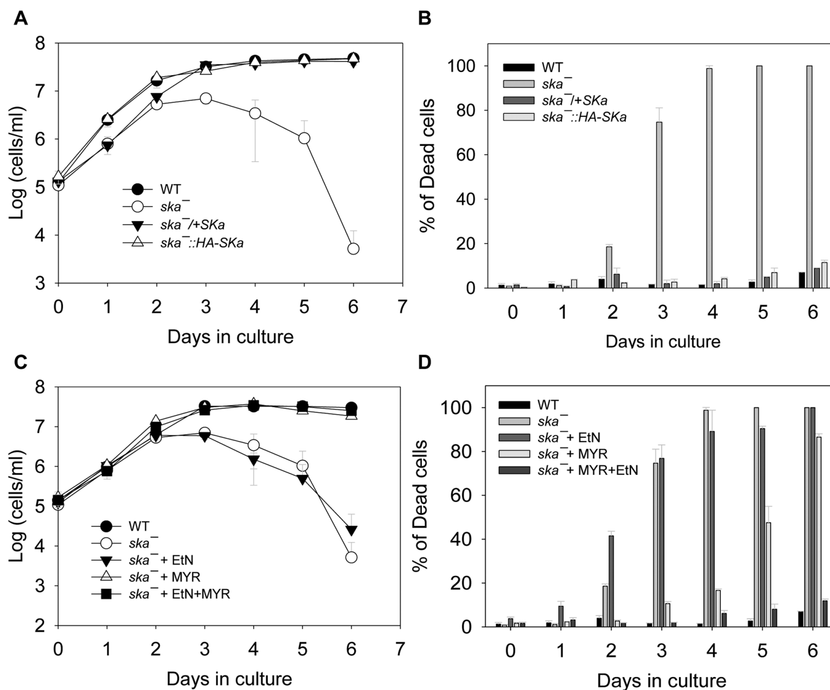

Fig. 3. Loss of SKa leads to catastrophic defects in culture due to sphingoid base accumulation. (A and B) *Ska⁻* mutants exhibit severe growth defects in culture. Promastigotes were inoculated at 1.0×10^5 cells ml^{-1} and incubated at 27°C. Culture densities (A) and percentages of dead cells (B) were monitored daily. (C and D) Rescue of *ska⁻* mutants by MYR + EtN. *Ska⁻* promastigotes were inoculated at 1.0×10^5 cells ml^{-1} in the presence of EtN (200 μM), MYR (1 μM) or EtN + MYR (200 and 1 μM respectively) and incubated at 27°C. Culture densities (C) and percentages of dead cells (D) were monitored daily. These experiments were repeated 4–5 times and error bars represent standard deviations.

acidic pH and increased concentration of reactive oxygen species/reactive nitrogen species. To investigate if SKa was required for thermotolerance, *L. major* promastigotes were cultured to stationary phase at 27°C (the regular temperature for promastigotes) and then exposed to higher temperatures (33°C or 37°C). As illustrated in Fig. 5A, the majority of *ska⁻* mutants (55–74%) lost viability at 37°C in 24 h, whereas WT, *ska⁻::HA-SKa* and *ska⁻/ + SKa* parasites were mostly alive (only 14–22% death). Meanwhile, *ska⁻* mutants survived much better after exposure to 33°C (Fig. 5A). To test whether SKa was involved in acid resistance, stationary phase promastigotes were transferred from a neutral medium (pH 7.4, the regular pH for promastigote medium) to an acidic medium (pH 5.0). Significantly, *ska⁻* mutants failed to adapt and 66–73% of them died within 24 h (Fig. 5B). In contrast, only 4–12% of WT and *ska⁻/ + SKa* cells were dead following the same treatment (Fig. 5B). We also evaluated the ability of *ska⁻* mutants to survive oxidative and nitrosative stress. As shown in Fig. 5C and D, these knockout parasites were hypersensitive to *S*-nitroso-*N*-acetylpenicillamine (a nitric oxide releaser) and hydrogen peroxide. Therefore, although *ska⁻* mutants supplemented with MYR and EtN could replicate and differentiate normally in culture, they were highly susceptible to stress.

As a control, WT promastigotes grown in the presence of MYR and EtN did not exhibit any significant difference in those tests (Fig. S10), indicating that the stress response defect of *ska⁻* is not caused by MYR and EtN (Fig. 5). In addition, exogenous sphingoid bases or ceramides had little effect on WT parasites in stress response (Fig. S10). Together, these findings suggest that it is the production of S1P (not the accumulation of sphingoid base) that is crucial for *Leishmania* to adapt to conditions in the mammalian host.

Surface virulence factors in *Leishmania* including lipophosphoglycan (LPG) and glycoprotein 63 (GP63) are known to confer resistance to oxidants, complements, and alter host signalling pathways (Spath *et al.*, 2003; Gomez *et al.*, 2009). To examine whether SKa played a role in the synthesis of GPI-anchored molecules, promastigote lysates were subjected to Western-blot analysis using antibodies against LPG and GP63. As illustrated in Fig. S11, cellular levels of LPG and GP63 in *ska⁻* and *ska⁻/ + SKa* were comparable to those in WT parasites, suggesting that deletion or overexpression of *SKa* had no adverse effect on the expression of GPI-anchored surface virulence molecules.

Ska⁻ and ska⁻/ + SKa parasites fail to cause disease in susceptible mice while the ska⁻::HA-SKa parasites are fully infective

The impact of SKa on *L. major* virulence was evaluated by macrophage infection and footpad infection of susceptible BALB/c mice. As shown in Fig. 6A and B, stationary phase *ska⁻* promastigotes (complemented with MYR + EtN) were able to invade and survive in murine macrophages (derived from the bone marrow of BALB/c mice) at a similar level as WT and *ska⁻/ + SKa* parasites. Apparently, *ska⁻*'s deficiency in stress response (summarized in

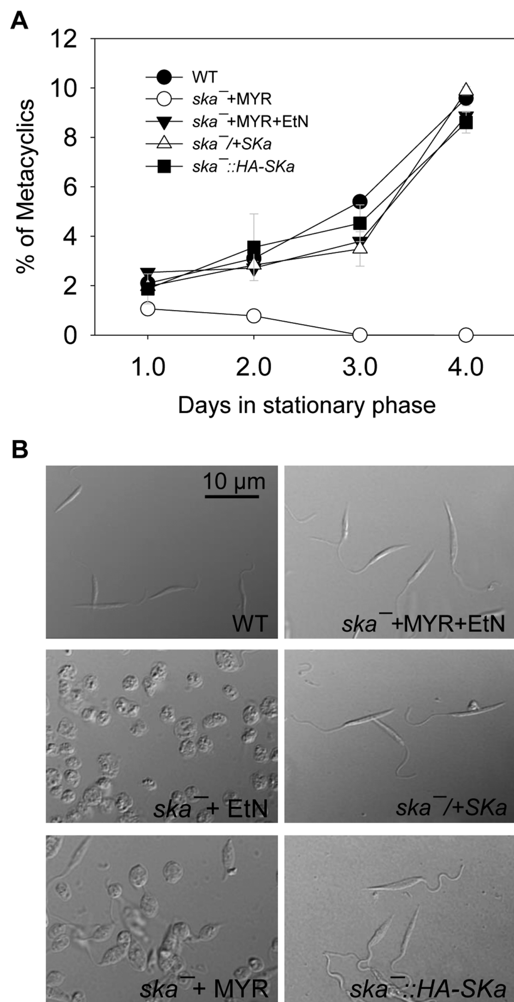

Fig. 4. *Ska*⁻ mutants supplemented with MYR and EtN show normal metacyclogenesis.
A. Percentages of metacyclics were determined daily from stationary phase cultures of WT, *ska*⁻ (supplemented with 1 μM of MYR only or 1 μM of MYR + 200 μM of EtN), *ska*⁻/ + SKa, and *ska*⁻*::HA-SKa* using the density centrifugation method. Experiments were performed in triplicates and error bars represent standard deviations.
B. Representative differential interference contrast images of cells from day 3 stationary phase cultures.

Fig. 5) does not affect their ability to parasitize macrophages in this short *in vitro* assay (3–4 days).

However, these *ska*⁻ mutants (stationary phase promastigotes) failed to generate any pathology in BALB/c mice (after 20 weeks), which were highly susceptible to WT infection (Fig. 7A). Curiously, although the *ska*⁻/ + *SKa* parasites (the episomal add-back) were completely normal as promastigotes in culture, they did not reverse the virulence defect of *ska*⁻ (Fig. 7A). Only the *ska*⁻*::HA-SKa* cells (the chromosomal knockin add-back) induced progressive footpad lesions (similar to WT) which correlated with the increasing number of parasites (Fig. 7A and B). Very similar results were obtained when mice were infected with purified metacyclics of WT, *ska*⁻ or *ska*⁻/ + *SKa* (Fig. S12A). Despite their lack of virulence, both *ska*⁻ and *ska*⁻/ + *SKa* parasites were able to persist at low levels in mice (1–600 parasites/footpad) (Fig. 7B and Fig. S12B). Finally, parasites recovered from *ska*⁻/ + *SKa*-infected mice (after 5–20 weeks) still contained the pXG–*SKa* episome (data not shown), ruling out the possibility of plasmid loss. The fact that only the chromosomal knockin add-back restores virulence suggests that in order to be functional during the mammalian stage, SKa must be expressed at near-WT level and/or from its endogenous locus.

Discussion

Previous studies of the *spt2*⁻ and *spl*⁻ mutants demonstrate that a major role of sphingoid base metabolism in *Leishmania* is to provide EtN-P, which is probably used to synthesize PE and PC (Zhang *et al*., 2007). Here we investigated the function of SK in *Leishmania* using reverse genetic approaches. SKs are conserved lipid kinases catalysing a key step in SL metabolism, i.e. the formation of S1P or DHS1P from Sph or DHS (Fig. S1). While most organisms possess two closely related SK isoforms, only one SK homologue (SKa) can be identified in *Leishmania* genome. Overexpression of *SKa* in *L. major* led to increased SK activity but no obvious defects in culture. *SKa*-null mutants (*ska*⁻) showed reduced SK activity but could still metabolize serine into downstream products including ceramide, IPC, PE and PC (Fig. 1). Remarkably, this partial block of SK activity was sufficient to cause a devastating consequence to *ska*⁻ promastigotes, as these mutants only grew to 1/4 to 1/3 of WT-level in culture and then died in 3–4 days (Figs 3 and 4). Further studies indicate that this catastrophic defect was likely due to the accumulation and release of toxic sphingoid bases generated from *de novo* synthesis (Figs 3 and 4; Figs S7–S9). To alleviate this problem, MYR and EtN were supplemented to *ska*⁻ to inhibit sphingoid base synthesis without compromising EtN-P production. Our results suggest that during the replicative log phase, *Leishmania* promastigotes must maintain a robust level of SKa to: (i) fulfil the need for EtN-P production and phospholipid synthesis; and (ii) eliminate free sphingoid bases to avoid cytotoxicity.

Besides detoxification, SKa also plays an important role in the stress response of non-replicative, stationary phase promastigotes. While *ska*⁻ promastigotes supplemented with MYR + EtN replicated and differentiated normally in culture, they were hypersensitive to heat, acidic pH, oxidants and nitrosative stress (Fig. 5). These defects are not caused by the accumulation of MYR, EtN, sphingoid bases, or ceramides (Fig. S10). Therefore, in addition to being intermediates for EtN-P production, S1P or DHS1P

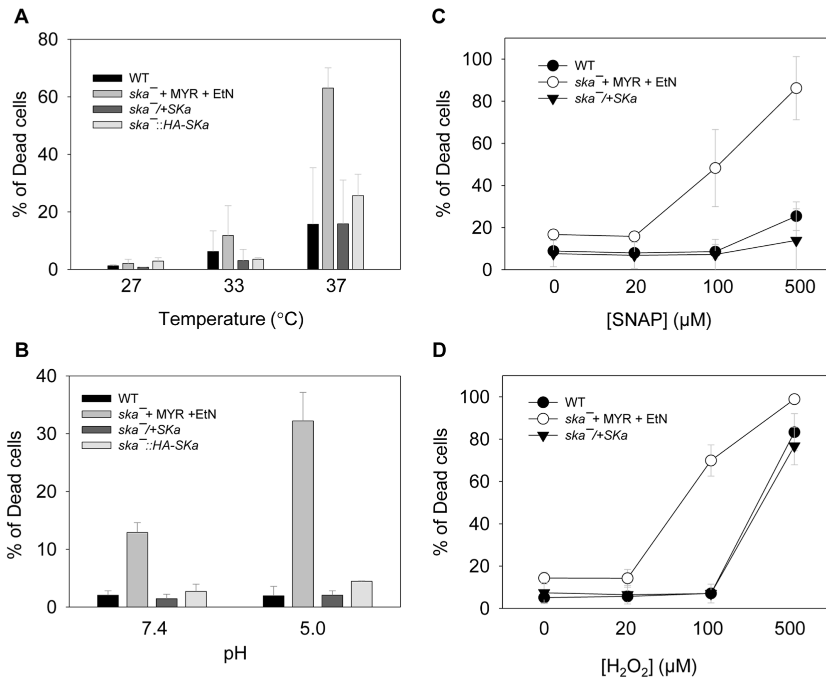

Fig. 5. *Ska*⁻ mutants are hypersensitive to stress conditions. (A and B) Promastigotes were cultured under regular condition (27°C, pH 7.4) to stationary phase before stress treatments. In A, parasites were transferred to 37°C, 33°C, or remained at 27°C. In B, half of the parasites were transferred to an acidic medium (pH 5.0) while the other half remained in regular medium (pH 7.4) for 72 h at 27°C. (C and D) Stationary phase promastigotes were challenged with various concentrations of SNAP (C) or hydrogen peroxide (D). In all experiments, *ska*⁻ promastigotes were complemented with 1 μM of MYR + 200 μM of EtN; percentages of cell death were determined at 24 h (A), 48 h (C and D), or 72 h (B) post treatments; and error bars indicate standard deviations from three independent repeats.

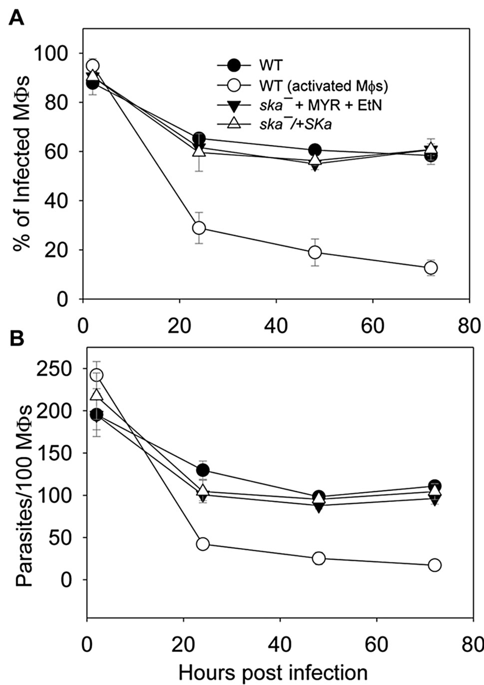

Fig. 6. *Ska*⁻ mutants supplemented with MYR and EtN survive well in MΦs *in vitro*. (A and B) Stationary phase promastigotes were used to infect bone-marrow derived MΦs (from BALB/c mice) as described in *Experimental procedures*. Percentage of infected MΦs (A) and the number of parasites in 100 MΦs (B) were recorded at 2, 24, 48 and 72 h post infection. As a control, WT promastigotes were also used to infect activated MΦs (pre-stimulated with 100 ng ml⁻¹ of LPS and 100 ng ml⁻¹ of IFN-γ). Error bars represent standard deviations.

may act as central regulators controlling the transition of *Leishmania* from insect stage to mammalian stage. In mammals, S1P exerts its function by binding to cell membrane receptors or intracellular targets. The cytoplasmic localization of SKa (in both promastigotes and amastigotes) suggests that the target of S1P/DHS1P in *Leishmania* is cytosolic, which is consistent with the lack of G-protein coupled membrane receptors in the annotated genomes of trypanosomatids. It is worth mentioning that the *spt2*⁻ mutants or WT parasites grown in the presence of MYR (defective in the *de novo* synthesis of sphingoid bases) did not exhibit any deficiency in resistance to heat, acidic pH, or oxidative/nitrosative stress when grown in the presence of EtN (data not shown). These findings suggest that *Leishmania* parasites can salvage sufficient amount of sphingoid bases to satisfy the need for S1P or DHS1P in stress tolerance. Alternatively, SKa protein may possess a secondary function that is vital for stress response yet independent of SK activity, which may explain its significant sequence divergence from other SKs (Fig. S2). Future studies will attempt to identify the proteins that bind SKa and/or its products (S1P/DHS1P), which may lead to new light on the role of sphingolipid metabolites in a protozoan parasite.

Although the *ska*⁻ mutants (when supplemented with MYR + EtN) could invade murine macrophages and survive well for 3–4 days *in vitro*, they completely failed to proliferate or cause disease in BALB/c mice (Figs 6 and 7). Such a severe virulence defect is in line with their inability to adapt to mammalian conditions. While the accumulation of sphingoid bases in *ska*⁻ is clearly detri-

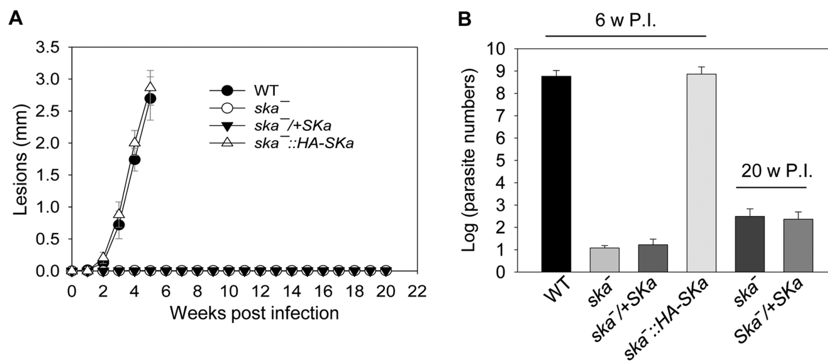

Fig. 7. *Ska⁻* and *ska⁻/ + SKa* parasites fail to cause pathology in BALB/c mice while the chromosomal knockin (*ska⁻::HA-SKa*) is fully virulent. BALB/c mice were infected in the footpads with stationary phase promastigotes (1.0×10^6 cells per mouse, 5 mice per group).
A. Lesion sizes of infected footpads were measured weekly with a caliper.
B. Parasite numbers in the infected footpads were determined at 6 or 20 weeks post infection (w P.I.) by limiting dilution assay.

mental to promastigotes, it is unlikely to be a major factor during the mammalian stage because the *de novo* synthesis of sphingoid base is greatly downregulated in amastigotes (which acquire EtN through salvage) (Zhang *et al*., 2005; 2007). Interestingly, only the chromosomal knockin add-back displayed WT-level infectivity while the episomal add-back failed to produce any pathology in mice (Fig. 7), despite the fact that both add-back parasites showed WT-like phenotypes in culture, e.g. in growth and stress response. It is possible that the flanking sequences of *SKa* open reading frame play a crucial role in the post-transcriptional regulation of SKa during the amastigote stage, such as ensuring mRNA stability and translation efficiency. This type of gene expression control is not uncommon among trypanosomatids (McNicoll *et al*., 2005; Haile *et al*., 2008) and may explain why *SKa* must be at its endogenous locus to be fully functional. Alternatively, the over expression of SKa may have deleterious consequences in the episomal add-back parasites during the amastigote stage (although it is well tolerated by promastigotes). In mammals, the synthesis of S1P is tightly regulated both spatially and temporally to limit any potential adverse effects from this powerful signalling molecule (Strub *et al*., 2010; Pitson, 2011; Siow *et al*., 2011). Further studies will shed light on the regulation and target(s) of SKa, which should expand our current knowledge on sphingoid metabolism in non-mammalian systems.

In summary, SKa is an extraordinarily important enzyme in both the promastigote stage and amastigote stage of *Leishmania* because: (i) a robust level of SKa is necessary to remove toxic sphingoid base from replicating promastigotes which have a high demand for EtN-P; (ii) SKa is also required the non-replicating promastigotes to survive elevated temperature, acidic pH and increased concentrations of reactive oxygen intermediates/reactive nitrogen intermediates; and (iii) the expression of SKa must be carefully regulated during the mammalian stage to ensure amastigote proliferation and disease progression. Since SKa is only marginally related to human SKs, it is tempting to consider its potential as a drug target. While even a partial inhibition of SK is detrimental to *Leishmania*, genetic deletion of either SK1 or SK2 leads to no obvious defects in mice and only the double knockout is lethal (Mizugishi *et al*., 2005). Therefore, mammals may be more tolerant to SK inhibitors than *Leishmania*. It is also of note that human SKs have been explored as targets for immunomodulatory drugs (e.g. FTY720) and anti-tumour drugs (Shida *et al*., 2008; Pitman and Pitson, 2010; Pyne and Pyne, 2011; Raje *et al*., 2012), using fluorescence-based, high throughput SK assays that are commercially available. In the future, to evaluate its potential as a new anti-*Leishmania* target, it is necessary to purify recombinant SKa. Although SKa is predicted to be a large protein (101 kDa), the lack of transmembrane domains and its cytoplasmic localization suggest the recombinant enzyme can be soluble.

Given the importance of SKa, future studies will also focus on other enzymes involved in the dynamics of sphingoid bases and S1P. For instance, the phenotype of *ska⁻* mutants suggests that although a second SK exists in *Leishmania*, it is not sufficient to detoxify sphingoid bases or control stress response when it is expressed at the endogenous level. While most organisms (including human, slime mould, worms, fruit fly and yeast) possess two isoforms of SK that share a high degree of sequence similarity (Hait *et al*., 2006), the second SK cannot be readily identified in the genomes trypanosomatid parasites. Future investigations may reveal why these protozoans produce two highly divergent SKs in the context of enzymatic specificity, parasite physiology and evolution. Another enzyme of interest would be the S1P phosphatase which catalyses the degradation of S1P. Along with the S1P lyase (SPL), S1P phosphatase counters the activity of SK by reducing S1P level therefore controls the cellular concentrations of sphingoid bases and their phosphorylated products. Characterization of enzymes involved in sphingoid base metabolism would improve our understanding on *Leishmania* biology and expand our knowledge on SL-mediated signalling.

Experimental procedures

Ethics statement for mouse use

The use of mice in this study was approved by the Animal Care and Use Committee at Texas Tech University (US PHS Approved Animal Welfare Assurance NO. A3629-01). BALB/c mice (female, 7–8 weeks old) were purchased from Charles River Laboratories International. Mice were housed and cared for in the facility operated by the Animal Care and Resources Center at Texas Tech University adhering to the *Guide for the Care and Use of Laboratory Animals* (the 8th Edition, NRC 2011) for animal husbandry. The facility was inspected monthly and animals were monitored daily by staff members. A complete range of clinical veterinary services was available on a 24 h basis including consultation, diagnostic work-up and clinical care. Lab personnel participating in this study are trained to use proper restraining and injection techniques to reduce pain and distress of animals without compromising the quality of research. Mice were anesthetized (through the intraperitoneal injection of 100 mg kg^{-1} of ketamine hydrochloride and 10 mg kg^{-1} of xylazine) prior to the injection of *Leishmania* parasites into footpads, the recovery of parasites from infected mice, and the measurement of lesion size using a caliper. Usually, no more than one procedure was performed on any mouse within a week. Mice were monitored twice a week for appearance, size, movement and general health condition. Mice were euthanized by carbon dioxide asphyxiation when footpad lesions became over 2.5 mm or when secondary infection was detected. Mice were also euthanized prior to the isolation of femur cells and limiting dilution assay (to determine parasite load in infected tissue).

Materials

Omega(7-nitro-2-1,3-benzoxadiazol-4-yl) (2S,3R,4E)-2-aminooctadec-4-ene-1,3-diol (C18 NBD-sphingosine) and omega(7-nitro-2-1,3-benzoxadiazol-4-yl) (2S,3R)-2-aminooctadecane-1,3-diol (C18 NBD-dihydrosphingosine) were purchased from Avanti Polar Lipids (Birmingham, AL). Human recombinant SK2 and ATP were purchased from Sigma-Aldrich. L-[3-^{3}H(G)]-serine (23Ci $mmol^{-1}$) was purchased from Perkin Elmers. All other chemicals were purchased from VWR International or Fisher Scientifics unless specified otherwise.

Molecular constructs

The open reading frame (ORF) of *SKa* (LmjF26.0710) was amplified by PCR from *L. major* genomic DNA using primers P207 (attactggatccaccATGCACTCTCTCAGCTCC) and P210 (attactggatccCTACCCACTGCGCACGAACTG). The resulting 2.8 kb DNA fragment was digested with BamHI and cloned into the pXG vector (Ha *et al.*, 1996) as pXG–*SKa* (B195). After confirming its sequence, the *SKa* ORF was subcloned into the pXG–GFP+2′ vector (Ha *et al.*, 1996) to generate pXG–GFP–*SKa* (B285), which was used in localization studies. To generate *SKa* knock-out constructs, the predicted 5′- and 3′-flanking sequences (~ 800 bp each) were PCR amplified and cloned in tandem into the pUC18 vector. Genes conferring resistance to puromycin (*PAC*) and blasticidin (*BSD*) were then inserted between the 5′- and 3′-flanking sequences to generate pUC-KO-*SKa:PAC* (B225) and pUC-KO-*SKa:BSD* (B224) respectively. In addition, a HA-tagged *SKa* ORF and its 5′-flanking sequence were cloned into the pGEM-phleo-HA′ (B202) vector (Madeira da Silva *et al.*, 2009) to generate pGEM-5′UTR-phleo-HA-*SKa* (B289). This construct was used to express *SKa* from its chromosomal locus (Madeira da Silva *et al.*, 2009).

Leishmania culture and genetic manipulations

Leishmania major FV1 (MHOM/IL/81/Friedlin) wild type (WT) promastigotes were grown in M199 medium with 10% fetal bovine serum (FBS) and other supplements as previously described (Kapler *et al.*, 1990). Unless specified otherwise, *ska*$^{-}$ mutants were grown in the presence of 1 μM of MYR and 200 μM of EtN. Treatment with MYR and EtN had no detectable effect on the proliferation, differentiation, or infectivity of WT parasites (data not shown). Promastigote replication was determined by monitoring culture density over time using a haemacytometer. Cell viability was examined by flow cytometry (using an Accuri C6 flow cytometer) after staining with 5 μg ml^{-1} of propidium iodide. Metacyclics were isolated from the stationary phase culture by the density gradient centrifugation method (Spath and Beverley, 2001) or the peanut agglutination method (Sacks *et al.*, 1985). The two *SKa* alleles in WT parasites were sequentially replaced by *PAC* and *BSD* resistance genes to generate the *ska*$^{-}$ mutant (*ΔSKa::PAC /ΔSKa::BSD*). Transfection and selection of drug-resistant promastigotes were performed as previously described (Kapler *et al.*, 1990). To confirm *SKa* deletion in *ska*$^{-}$, genomic DNA was digested with ScaI, resolved on a 0.7% agarose gel, transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labelled DNA probe corresponding to either the *SKa* ORF or the 5′-flanking sequence. To restore SKa expression via episomal overexpression, pXG–*SKa* was transfected into the mutant and the resulting add-back parasite (resistant to G418) was referred to as *ska*$^{-}$/ + *SKa* (*ΔSKa::BSD/ΔSKa::PAC+* pXG–*SKa*). To restore SKa expression to near WT-level, we generated a *SKa* chromosomal knockin by integrating a HA-tagged *SKa* ORF into the genomic locus of SKa as previously described (Madeira da Silva *et al.*, 2009). To do so, the plasmid DNA of pGEM-5′UTR-phleo-HA-SKa was linearized with EcoRI and introduced into the *SKa* heterozygote parasites (*ΔSKa::BSD/SKa*); cells showing resistance to blasticidin and phleomycin were selected; finally, these knockin parasites were confirmed by Southern-blot and designated as *ska*$^{-}$*::HA-SKa* (*ΔSKa::BSD/ΔSKa::HA-SKa*). To study SKa localization, pXG–GFP–*SKa* was transfected into WT parasites to generate WT/ + GFP–SKa parasites (resistant to G418).

SK activity assay

Log phase promastigotes (1–4 × 10^{6} cells ml^{-1} in culture) were resuspended in a lysis buffer (50 mM HEPES pH 7.4, 800 mM KCl, 2 mM dithiothreitol, 2 mM ATP, 0.02% Triton X-100, 20 mM $MgCl_2$, 20 mM NaF and 2× protease inhibitor

cocktail) at 4.0×10^8 cells ml^{-1} and incubated on ice for 5 min. Protein concentration was determined by the BCA assay (Pierce). SK assay was performed as previously described with minor modifications (Billich and Ettmayer, 2004). Briefly, C18 NBD-Sph or C18 NBD-DHS was prepared in 4 mg ml^{-1} low fat bovine serum albumin at a final concentration of 30 μM. Each assay reaction contained 25 μl of *Leishmania* cell lysate (~ 40 μg of total protein) and an equal volume of C18 NBD-Sph or C18 NBD-DHS. After 30 min incubation at 27°C, the reaction was terminated by adding 50 μl of K_2HPO_4 (1 M, pH 8.5) and 250 μl of chloroform : methanol (2:1). After centrifugation (13 000 *g*, 2 min), the upper aqueous phase (containing NBD-labelled S-1-P or DHS-1-P) was separated from the lower organic phase (containing NBD-labelled substrates). Fractions of upper phase and lower phase were loaded separately on aluminum-backed silica gel plates (Sigma-Aldrich). NBD-labelled products/substrates were separated by thin layer chromatography (solvent: chloroform/methanol/ammonium hydroxide = 10/10/3) and detected with a Storm 860 phosphoimager. 0.1 unit of recombinant human SK2 was used as a positive control and boiled WT lysate (~ 40 μg of total protein) was used as a negative control. SK activity [nmol $(\mu g \times h)^{-1}$] was calculated based on the amount of NBD S1P or NBD DHS1P produced in each reaction after subtracting the value of negative control.

Metabolic labelling with [3-^{3}H]-serine

Promastigotes were inoculated at 1.0×10^5 cells ml^{-1} and labelled with 1.5 μCi ml^{-1} of L-[3-^{3}H(G)]-serine. After 48 h, parasites were harvested and total lipids (from ~ 1×10^8 cells) were extracted using the Bligh-Dyer method (Bligh and Dyer, 1959). TLC was performed as previously described (Zhang *et al*., 2007).

Lipid analysis by electrospray ionization mass spectrometry (ESI-MS)

Lipid extraction and analysis by ESI-MS (negative ion mode) were performed similarly as previously described (Zhang *et al*., 2007). A 1-*O*-1′-(Z)-octadecenyl-2-arachidoyl-*sn*-glycero-3-phosphoethanolamine (*p*18:0/20:4-PE, FW = 751.6, Avanti Polar Lipids) was used as a standard to quantify PLE. This standard was added to cell lysates prior to lipid extraction (at 1.0×10^8 molecules per cell).

Immunofluorescence microscopy

To determine the localization of SKa in promastigotes, log phase parasites of WT/ + GFP–*SKa* or *ska*$^-$*::HA-SKa* were fixed in 3.7% formaldehyde, attached to poly-L-lysine coated coverslips, and permeabilized with 0.5% Triton X-100 (prepared in PBS) at room temperature for 15 min. Immunofluorescence microscopy was performed as previously described (Zhang *et al*., 2003). Primary antibody: rabbit anti-GFP polyclonal antibody (1:1000, Life Technologies) or rabbit anti-HA polyclonal antibody (1:50, Life Technologies). Secondary antibody: goat anti-rabbit IgG-Texas Red (1:1000, Life Technologies).

To determine the localization of SKa in amastigotes, THP-1 derived macrophages were infected with stationary promastigotes (10 parasites per macrophage) and incubated at 37°C/5% CO_2 for 48 h. Macrophages were then fixed in 3.7% formaldehyde and permeabilized with ice-cold ethanol for 15 min. Immunofluorescence assay was then performed as described above.

Leishmania stress response

Promastigotes were grown in regular medium (M199/10% FBS and supplements, pH 7.4) at 27°C to stationary phase (culture density = 2.0–3.5×10^7 cells ml^{-1}) and used in the following assays. To test temperature sensitivity, parasites were kept in regular medium and incubated at 27°C, 33°C/5% CO_2, or 37°C/5% CO_2 (Xu *et al*., 2011). To examine their tolerance to acidic pH, parasites were either kept in regular medium (pH 7.4) or transferred to a pH 5.0 medium (same as the regular medium except the pH was modified with hydrochloric acid) and incubated at 27°C (Xu *et al*., 2011). To measure their sensitivity toward oxidative and nitrosative stress, parasites were cultured in 24-well plates (regular medium, 27°C) in the presence of 0–500 μM of H_2O_2 or *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Xu *et al*., 2011). Cell density and viability were determined at the indicated time points.

Murine macrophage (MΦ) infection

Bone marrow-derived MΦs were generated from the femur cells of BALB/c mice in complete DMEM/10% FBS + 20 ng ml^{-1} macrophage colony-stimulating factor for 4 days at 37°C/5% CO_2. Infection was performed with late stationary phase parasites (opsonized with C57BL6 mouse serum) at a ratio of twenty parasites per one MΦ as previously described (Racoosin and Beverley, 1997). MΦs were then incubated at 33°C/5% CO_2 for 3 days. Percentages of infected MΦs and the number of parasites per 100 MΦs were determined by microscopy after staining with 1 μg ml^{-1} of Hoechst 33342.

Mouse infections and analyses

Stationary phase promastigotes were inoculated into BALB/c mice (2–5×10^7 cells per mouse) and recovered after 3–4 weeks. Virulence of these low passage parasites (*in vitro* passages < 5) was evaluated by footpad infection as previously described (Titus *et al*., 1991), using either stationary promastigotes (1×10^6 cells per mouse, 5 mice per group) or purified metacyclics (2×10^5 cells per mouse, 5 mice per group). Footpad lesions were measured weekly using a vernier caliper and parasite numbers were determined by limiting dilution assay (Titus *et al*., 1985).

Western blot

To determine the expression level of GPI-anchored molecules, promastigotes were harvested and Western blot analyses were performed with antibodies against LPG or GP63 as previously described (Zhang *et al*., 2004).

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