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Sphingolipid synthesis and scavenging in the intracellular apicomplexan parasite, *Toxoplasma gondii*

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ABSTRACT

Sphingolipids are essential components of eukaryotic cell membranes, particularly the plasma membrane, and are involved in a diverse array of signal transduction pathways. Mammals produce sphingomyelin (SM) as the primary complex sphingolipid *via* the well characterised SM synthase. In contrast yeast, plants and some protozoa utilise an evolutionarily related inositol phosphorylceramide (IPC) synthase to synthesise IPC. This activity has no mammalian equivalent and IPC synthase has been proposed as a target for anti-fungals and anti-protozoals. However, detailed knowledge of the sphingolipid biosynthetic pathway of the apicomplexan protozoan parasites was lacking. In this study bioinformatic analyses indicated a single copy orthologue of the putative SM synthase from the apicomplexan *Plasmodium falciparum* (the causative agent of malaria) was a *bona fide* sphingolipid synthase in the related model parasite, *Toxoplasma gondii* (*Tg*SLS). Subsequently, *Tg*SLS was indicated, by complementation of a mutant cell line, to be a functional orthologue of the yeast IPC synthase (AUR1p), demonstrating resistance to the well characterised AUR1p inhibitor aureobasidin A. *In vitro*, recombinant *Tg*SLS exhibited IPC synthase activity and, for the first time, the presence of IPC was demonstrated in *T. gondii* lipid extracts by mass spectrometry. Furthermore, host sphingolipid biosynthesis was indicated to influence, but be non-essential for, *T. gondii* proliferation, suggesting that whilst scavenging does take place *de novo* sphingolipid synthesis may be important for parasitism.

1. Introduction

Toxoplasma gondii is an obligate, intracellular protozoan parasite, which is able to invade and colonise a wide variety of nucleated vertebrate cells. It is a member of the Apicomplexa, a diverse phylum including important pathogens of humans and domestic animals such as *Plasmodium* (the causative agent of malaria), *Cryptosporidium* (diarrhoea), *Eimeria* (coccidiosis in poultry) and *Theileria* (East Coast Fever in cattle). *Toxoplasma* has emerged as an opportunistic pathogen and toxoplasmosis is an important disease in the immunocompromised, particularly AIDS patients, those receiving anti-cancer chemotherapy and organ transplant recipients [1]. *Toxoplasma* infection *in utero* is also a significant cause of congenital defects in humans [1] and spontaneous abortion in economically important domestic animals [2].

Sphingolipids are amphipathic lipids comprising sphingosine as the basic building unit. More complex sphingolipids consist of a sphingosine backbone *N*-acylated with a long-chain fatty acid (*i.e.* ceramide) and substituted with a head group moiety (*e.g.* sphingomyelin, glucosylceramide and ceramide-1-phosphate) [3].

Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; IPC, inositol phosphorylceramide; SM, sphingomyelin; CPE, ceramide phosphorylethanolamine; NBD, C_6-ceramide-*N*-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-*erythro*-sphingosine; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; *Tg*SLS, *Toxoplasma gondii* sphingolipid synthase; MEF, mouse embryonic fibroblasts.

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Ceramide is a sphingolipid that functions as a secondary messenger in ubiquitous, evolutionarily conserved, signalling mechanisms [4]. Complex sphingolipids are major components of the outer leaflet of eukaryotic plasma membranes that are thought to be involved, together with sterols, in the formation of micro-domains known as lipid rafts. These rafts have been proposed to function in a diverse array of processes from the polarised trafficking of lipid-modified proteins, to the assembly and activation of signal transduction complexes [5]. In the apicomplexan *Plasmodium* species, sphingolipid-enriched lipid rafts have been implicated in the interaction of the parasite with the host erythrocyte through the trafficking of both host and parasite glycosylphosphatidylinositol (GPI) anchored proteins [6]. In addition, it has been demonstrated, by the incorporation of tritiated serine, that both *Plasmodium falciparum* and *T. gondii* synthesise sphingolipids *de novo* [7,8]. Like mammals, *P. falciparum* synthesises the complex phosphosphingolipid sphingomyelin (SM) [9–11] and an orthologue of the mammalian enzyme, SM synthase, has been identified from the genome database [12]. *T. gondii* has also been indicated to synthesise SM, although at relatively low levels compared to glycosphingolipids [8], and the presence of this species has subsequently been confirmed using mass spectrometry [13]. However, the enzyme responsible for any SM synthase activity has remained unidentified in *T. gondii*, and uncharacterised in any apicomplexan. Furthermore, it has also been reported that the parasites harbour relatively high quantities of ceramide phosphorylethanolamine (CPE), a non-abundant species in mammalian cells [13]. In addition, the synthesis of the non-mammalian phosphosphingolipid, inositol phosphorylceramide (IPC), has also been reported in *T. gondii* [14]. Importantly, the biosynthetic enzyme, IPC synthase, has been validated as a drug target in both the fungi and the kinetoplastid protozoa [15–18], and its inhibition by the anti-fungal aureobasidin A has been proposed in *T. gondii* [14].

Notably, in addition to *de novo* synthesis, intracellular parasites such as *T. gondii* may scavenge sphingolipids or their precursors from the host cell [19]. Indeed it has been suggested that the CPE (and SM) found in intracellular tachyzoites forms may result from the concentration of non-abundant host-derived lipid [13]. Within the host cell *T. gondii* resides within a specialised parasitophorous vacuole (PV) formed immediately after invasion and delineated by the PV membrane (PVM) [20]. Although the PV resists fusion with host organelles it does demonstrate an intimate, high affinity association with the ER and mitochondrion [21], the latter facilitating the scavenging of host lipoic acid [22]. Furthermore, recent work has indicated that host-derived lipid is the primary contributor to the intravacuolar network that fills the lumen of the PV [23]. *Toxoplasma* scavenges a variety of fatty acids and lipids from the host, including phospholipids and cholesterol, some of which are further metabolised by the parasite [24,25]. The mechanism of lipid scavenging is unclear, although current data argue against passive diffusion, acquisition on invasion [25] and (at least in the case of cholesterol) vesicular trafficking [24]. It has been proposed that the transport of cholesterol to the PV could be mediated *via* a protein carrier [24], and the possibility of direct inter-organelle transfer of lipids between the closely associated PVM and host ER and mitochondrial membranes has been evoked [22,25]. The balance between *de novo* synthesised and scavenged lipid is unclear, however when host phosphatidylcholine (PC) levels are restricted it is likely that the parasites scavenge choline and synthesise PC *de novo* [25].

To begin to understand the role of both *de novo* synthesis and scavenging of sphingolipid for *T. gondii*, we aimed to begin characterisation of the little understood parasite biosynthetic pathway and investigate the requirement, if any, for host sphingolipid. To these ends we herein report the identification and functional characterisation of a key enzyme in *T. gondii* sphingolipid synthesis that may represent a novel drug target and, in addition, show the delineation of the role of host biosynthesis in parasite proliferation.

2. Materials and methods

2.1. Selection, sequence analyses and cloning of candidate sphingolipid synthase

The *T. gondii* genome database (www.toxodb.org) was interrogated (Gish, 1996–2001) (http://blast.wustl.edu) with the two candidate sphingolipid synthase coding sequences previously identified from the genome database of the malaria parasite *P. falciparum* (plasmodb.org) [12]. A single sequence orthologue was identified, *Tg*SLS accession number TGME49_046490, corresponding to the entry previously identified [13]. Sequence alignments were made using ClustalW [26] and phylogenetic analyses performed on the edited alignments using Maximum Parsimony, Protein Distance (PHYLIP Phylogeny Inference Package, version 3.5c) and Maximum Likelihood [27]. The candidate *Tg*SLS open reading frame was amplified from genomic *T. gondii* DNA using *Pfu* polymerase (Promega) and the primer pair 5′TgSLSEcoRI (cgcgaattcATGCCCAGAACAGAGATG) and 3′*Tg*SLS*HindIII (cccaagcttTTAGAGTCCCTCGATGGCGCGAACGAT). Cloning sites shown in lower case, with coding sequence in upper case. The product was purified, digested and cloned into the yeast expression vector pRS426MET25 creating pRS426 *Tg*SLS.

2.2. Functional complementation of auxotrophic yeast AUR1 mutant

pRS426 *Tg*SLS, together with pRS426 AUR1 and empty vector, were used to transform the YPH499-HIS-GAL-AUR1 *Saccharomyces cerevisiae* strain [28]. Transformants were selected on non-permissive SD-HIS-URA medium (0.17% Bacto yeast nitrogen base, 0.5% ammonium sulphate and 2% dextrose) or permissive SGR-HIS-URA medium (0.17% Bacto yeast nitrogen base, 0.5% ammonium sulphate, 4% galactose and 2% raffinose) containing the appropriate nutritional supplements at 30 °C.

2.3. In vitro assay of TgSLS activity

Microsomal membranes from exponentially growing YPH499-HIS-GAL-AUR1 pRS426 *Tg*SLS or pRS426 AUR1 in SD-HIS-URA were prepared and the isolated membrane fraction re-suspended in storage buffer (50 mM Tris–HCl pH 7.4, 20% (v/v) glycerol, 5 mM $MgCl_2$) with Complete® EDTA-free Protease Inhibitor Cocktail (Roche Applied Science) at a protein concentration of 10 mg/ml as described previously [18]. Microsomal membranes were subsequently washed in 40 mM CHAPS (4 °C, 60 min), isolated by centrifugation (150,000 × *g*, 4 °C and 100 min), re-suspended in storage buffer at 10 mg/ml and stored at −80 °C until use. The assay mix contained 1 mM donor substrate (bovine liver PI, PC or PE, Avanti Polar Lipids), 20 μg microsome prep, 100 mM Tris–HCl, 10 mM EDTA, 6 mg/ml BSA and 5 μM NBD C_6-ceramide [19]. Following incubation at 30 °C for 60 min the reaction was quenched by the addition of 150 μl of chloroform:methanol:water (10:10:3) and lipids separated and analysed as above. For inhibition experiments the reaction mix was pre-incubated for 30 min with appropriate quantities of aureobasidin A (Takara Bio Inc.) before the addition of NBD C_6-ceramide.

2.4. Agar diffusion assay

YPH499-HIS-GAL-AUR1 complemented with *TgSLS* or AUR1 were assayed for susceptibility to aureobasidin A and myriocin (Sigma) as previously described [28]. Briefly, 2.4 OD_{600} units of