Sphingolipid Synthases of Toxoplasma gondii and other organisms

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THE SPHINGOLIPID SYNTHASES OF *TOXOPLASMA GONDII* AND OTHER ORGANISMS

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ABSTRACT

THE SPHINGOLIPID SYNTHASES OF *TOXOPLASMA GONDII* AND OTHER ORGANISMS

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Toxoplasma gondii is an obligate intracellular protozoan parasite of the phylum Apicomplexa, and toxoplasmosis is an important disease in immuno-compromised individuals - especially AIDS patients, organ transplant recipients and patients receiving anti-cancer chemotherapy. Toxoplasmosis also has economic importance as it results in spontaneous abortion in economically important animals. Due to emerging drug resistance and the possible side effects of the existing therapeutics there is a necessity to explore new drug targets. Sphingolipids are essential components of eukaryotic cell membranes and signal transduction pathways. Mammals produce sphingomyelin (SM) via a SM synthase, whereas yeast, plants and some protozoa utilise an inositol phosphorylceramide (IPC) synthase to produce IPC. IPC synthases have no mammalian equivalent and have been proposed as targets for anti-fungals and anti-protozoals. In this study sphingolipid scavenging from the host was shown to be non-essential for *Toxoplasma* proliferation indicating *de novo* synthesis is key. To investigate this pathway in the parasite an orthologue of the SM synthase from the related apicomplexan *Plasmodium falciparum* was identified in the genome database as being encoded by a single copy gene. Subsequently it was characterized as a functional orthologue of the yeast AUR1p (IPC synthase), with mass spectrometry identifying this lipid species in parasite extracts for the first time. Like AUR1p and the human SM synthase, the *Toxoplasma* sphingolipid synthase (*Tg* SLS) is Golgi localized. However, unlike AUR1p *Tg* SLS is resistant to the inhibitor aureobasidin A and also facilitates production of a minor complex sphingolipid suggested to be SM. Further study characterized the sphingolipid synthases from the *Trypanosoma* species (protozoan Kinetoplastida) and the plant *Arabidopsis.* Bioinformatic and phylogenetic analyses of these and previously characterized SM and IPC synthases, indicate them to be a family of evolutionarily related, but functionally diverse, enzymes. Many of these may prove to be targets for pharmaceutical or herbicidal intervention.

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LIST OF ABBREVIATIONS

Ab A	Aureobasidin A
AMP	Ampicillin
AUR1	Aueobasidin Resistance protein
***At* IPCS**	*Arabidopsis thaliana* Inositol Phosphoryl Ceramide Synthase orthologue
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
BSA	Bovine Serum Albumin
BSF	Bloodstream Form
C1P	Ceramide-1-phosphate
CER	Ceramide
CHAPS	3-[(3-Cholamidopropyl) Dimethylammonio]-1-Propanesulfonate
CHO	Chinese Hamster Ovary
C:M	Chloroform: Methanol (v:v)
C:M:W	Chloroform: Methanol: Water (v:v:v)
CoA	Co enzyme A
D	Domain
Da	Daltons
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DO	Drop Out
E. coli	*Escherichia coli*

EDTA	Ethylene Diamine Tetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
EPC	Ethanolamine Phosphoryl Ceramide
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FLC_5	FL C_5 Ceramide
g	Gram
x g	g-force
GAL	Galactose
GFP	Green Fluorescent Protein
GPI	Glycophosphatidylinositol
GIPLs	Glycoinositolphospholipids
GSL	Glycosphingolipids
GYT	Glycerol / Yeast extract / Peptone
[^{3}H]	Tritiated
h	Hours
HFF	Human Foreskin Fibroblasts
HIS	Histidine
HPTLC	High Performance Thin Layer Chromatography
***Hs* SMS**	(*Homo sapiens sapiens*) Human Sphingomyelin synthase orthologue
IPC	Inositol phosphorlyceramide
kDa	Kilo Dalton
KDS	3-ketodihydrosphingosine
kV	Kilo volt
L	Litre
LC-MS	Liquid Chromatography-Mass Spectrometry

LiAc	Lithium acetate
LPP	Lipid Phosphate Phosphotase
LPPs	Lipophosphotransferases
MEF	Mouse embryonic fibroblast
MET	Methionine
mg	Milli gram
min	Minutes
ml	Milli litre
mM	Milli molar
MS	Mass Spectrometry
ms	Milli second
Myr	Myriocin
nm	Nano meter
NBD C_6	N-[6-[7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-D-erythro-sphingosine
NCBI	National Centre for Bioinformatic Information
NH_4SO_4	Ammonium sulphate
NTD	Neglected tropical disease
NZY+	NZ amine mix and yeast extract medium
OD_{600}	Optical density at 600nm
PBS	Phosphate buffered saline
PC	Phosphatidyl Choline
PCF	Procyclic form
PCR	Polymer chain reaction
PDMP	*threo*-phenyl-2-decanoylamino-3-morpholino-1-propanol
PE	Phosphatidyl Ethanolamine

PEG	Polyethelyneglycol
P*fu*	DNA polymerase proof reading enzyme
pH	-Log [Hydrogen ion concentration]
PI	Phosphatidyl Inositol
PLB	Paul's Luria-Bertani broth
ppm	Parts per mass
PPMP	*threo*-phenyl-2-palmitoylamino-3-morpholino-1-propanol
PV	Parasitophorus vacuole
PVM	Parasitophorus vacuole membrane
RNA	Ribonucleic acid
RTPCR	Reverse Transcriptase PCR
***Sc* AUR1**	*Saccharomyces cerivisiae* inositol phosphoryl ceramide homolouge
SD	Synthetic dextrose medium
SGR	Synthetic galactose and raffinose medium
S1P	Sphingosine-1-phosphate
SL	Sphingolipids
SLS	Sphingolipid synthase
SMase	Sphingomyelinase
SMS	Sphingomyelin synthase
SPB-1	Sphingoid base biosynthesis mutant
SPT	Serine palmitoyltransferase
STE buffer	Sucrose / Tris / EDTA buffer
TAE	Tris Base / Sodium Acetate / EDTA buffer
***Tb* SLS**	*Trypanosoma brucei* Sphingolipid synthase orthologue
***Tc* SLS**	*Trypanosoma cruzi* Sphingolipid synthase orthologue
TE	Trisma / EDTA buffer

***Tg* SLS**	*Toxoplasma gondii* Sphingolipid synthase
TLC	Thin Layer Chromatography
TM	Transmembrane domain
Tris	Trisma
TRP	Tryptone
URA	Uracil
UV	Ultra violet
v	Volt
VERO Cells	Kidney epithelial cells from African Green Monkey
YNB	Yeast Nitrogen Base
YPD	Yeast / Peptone / Dextrose
YPGR	Yeast / Peptone / Galactose / Raffinose medium
2YT^{+}	Tryptone extract + NaCl
µCi	Micro Curies
µF	Micro Faraday
µl	Micro litre
µM	Micro molar
µm	Micro meter

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RESEARCH BACKGROUND

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2000-2004 BSc (Hons), (1st class) Special degree in Biological sciences including Chemistry, Faculty of Science, University of Colombo, Sri Lanka

Final year specialized in Immunoparasitology with research project on Immunology of Malaria: "***Development and testing of an ELISA to assay the natural antibody responses to the Duffy Binding Protein of Plasmodium vivax in Sri Lanka*".**

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1. **Comparison of the two different recombinant proteins representing region II of the Duffy Binding Protein of *Plasmodium vivax* by assaying for natural antibodies,** was published and presented at the proceedings of annual sessions of the Society of Allergy and Immunology Sri Lanka associated with FIMSA (Federation of Immunological Societies Asia Oceania) 19th November 2004.

2. **Human Natural Antibody Responses to the *Plasmodium vivax* Duffy binding Protein in Sri Lanka,** was published and presented at the annual proceedings of University of Colombo Joint (Faculties of Science and Medicine) Academic sessions 25th June 2004.

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Contributed to two international research publications (see appendices):

1. **Functional analyses of differentially expressed isoforms of the *Arabidopsis* Inositol phosphorylceramide synthase,** *Journal of Plant Molecular Biology* 2010 Jul; 73 (4-5): 399-407. Epub 2010 Mar 23.

2. **The *Trypanosoma brucei* sphingolipid synthase, an essential enzyme and a drug target**, *Journal of Molecular & Biochemical Parasitology* 2009 Nov; 168 (1):16-23. Epub 2009 Jun 21.

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DEDICATION

"This thesis is dedicated to my deceased father, Wansadhipathige Cyril Silva, whose endless love, enormous effort and courage of the 32 years of fatherhood honoured with great respect."

CHAPTER 01

INTRODUCTION

1 INTRODUCTION

1.1 *TOXOPLASMA GONDII*

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to phylum Apicomplexa (Cortex *et al*., 2007). It is also a highly ubiquitous and prevalent parasite with a widespread distribution both in terms of geographical and host range (Hide *et al*., 2009; Hide *et al*., 2007). *Toxoplasma gondii* is an opportunistic pathogen causing toxoplasmosis, which is an important disease in immuno-compromised individuals such as patients receiving anti-cancer chemotherapy, organ transplant recipients and AIDS patients (Chowdhury, 1986). It causes congenital defects in humans and spontaneous abortion in economically important and domestic animals (Huong and Dubey, 2007).

The significance of *Toxoplasma* as a model system for other disease-causing apicomplexan parasites including *Plasmodium*, the causative agent of malaria; *Eimeria*, which is the cause of poultry coccidiosis; and *Cryptosporidium*, which is another important opportunistic infection in AIDS patients, is emerging (Blader and Saeij, 2009).

1.2 TAXONOMY OF *TOXOPLASMA*

According to NCBI Entrez taxonomy (ncbi.nlm.nih.gov/),

Domain : Eukarya

Super Kingdom : Eukaryota

Kingdom : Chromalveolata

Super Phylum : Alveolata

Phylum : Apicomplexa

Class : Coccidia

Order : Eucoccidiorida

Sub Order : Eimeriorina

Family : Sarcocystidae

Genus : *Toxoplasma*

Species : *Toxoplasma gondii*

There are 7 species recognized in this genus: *Toxoplasma gondii* from about 200 species of mammals and birds; *Toxoplasma alencari* from the frog *Leptodactylus ocellatus; Toxoplasma brumpti* from the iguana *Iguana tuberculata; Toxoplasma colubri* from the snakes *Coluber melanoleucus* and *Coluber viridiflavus; Toxoplasma hammondi* (a new name for *Hammondia hammondi*) from the house mouse; *Toxoplasma ranae* from the leopard frog *Rana pipiens;* and *Toxoplasma serpai* from the toad *Bufo marinus* (Levine, 2008).

1.3 HISTORY OF THE ORGANISM

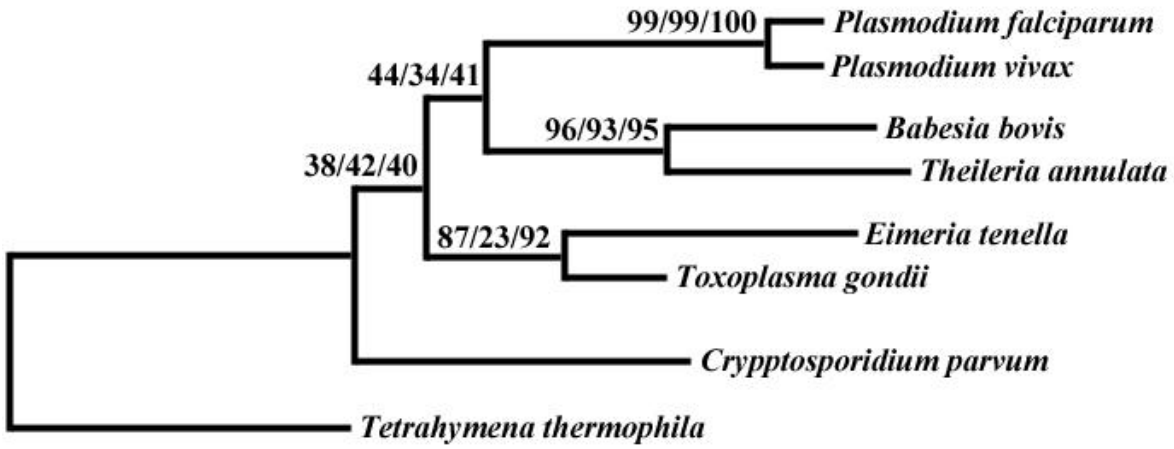
This organism was first described by Charles Nicolle and Louis Manceaux in the rodent *Ctenodactylus gundi* and by Alfonso Splendore in rabbits in 1908, and named a year later (Black and Boothroyd, 2000; Dubey, 2008). By 1937 very little was known about the nature of this organism and it was grouped with parasites of doubtful nature by Sabin and Olitsky (Manwell, 1941). Its medical importance remained unknown until 1939 when it was identified in tissues of a congenitally infected infant, and its veterinary importance became known when it was found to cause abortion storms in sheep in 1957 (Dubey, 2008).

Toxoplasma gondii was identified as small and usually crescent-shaped parasite and named based on its morphology. The species designation originated from the name of the North African rodent (*Ctenodactylus gundi*), and the genus name was derived from the Greek word toxon meaning "bow" and referring to the crescent shape of the organism (Black and Boothroyd, 2000). *T. gondii* occur in various types of leucocytes, body fluids and endothelial cells (Manwell, 1941) and can be found in large numbers in various organs such as liver, spleen, bone marrow, lungs, kidneys and brain. It was also identified as infecting a variety of hosts, including mammals and birds (Manwell, 1941).

1.4 POSITION OF *TOXOPLASMA* IN APICOMPLEXAN PHYLOGENY

The Apicomplexa are a group of phylogenetically related parasitic protists. Based upon the genome sequences from seven apicomplexan species and a ciliate outgroup it has been found that the piroplasms *Theileria* and *Babesia* form the sister group to the *Plasmodium* species, the coccidian *Eimeria* and *Toxoplasma* are monophyletic and are the sister group to the *Plasmodium* species and piroplasms. *Cryptosporidium* forms the sister group to the above (Figure 1-1; Kuo, Wares and Kissinger, 2008).

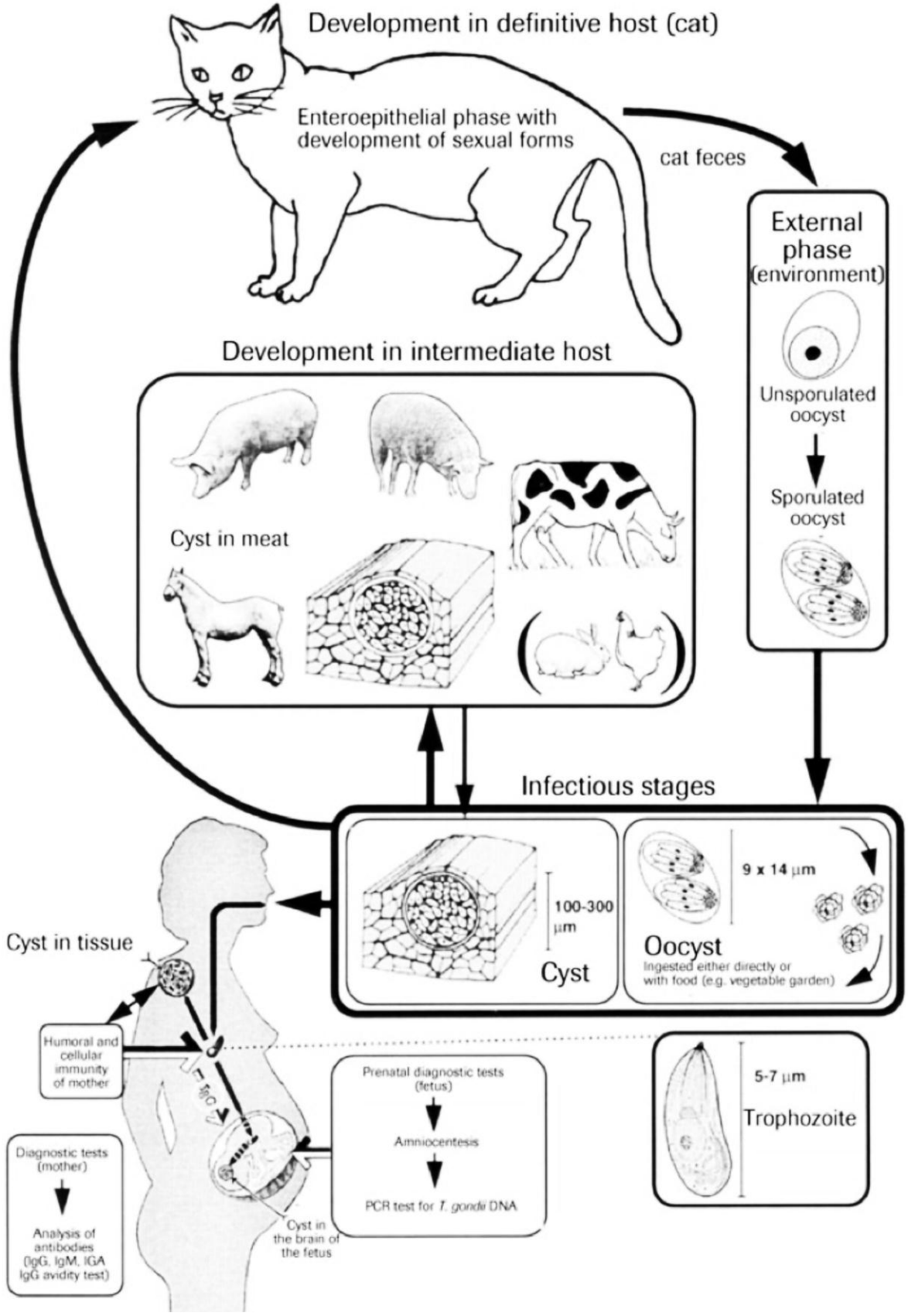
Figure 1-1: An inferred apicomplexan species tree. The Maximum Likelihood (ML) tree is generated from the concatenated alignment of 268 single-copy genes. Labels above branches indicate the level of consensus support (%) based on ML, Maximum Parsimony (MP) and Neighbour Joining (NJ) (Kuo, Wares and Kissinger, 2008).



1.5 LIFE CYCLE AND TRANSMISSION

The life cycle of *Toxoplasma gondii* was not discovered until 1970 when it was found that felids are its definitive host and an environmentally resistant stage i.e. oocyst is excreted in faeces of infected cats (Dubey, 2008).

Figure 1-2: Routes of transmission and life cycle of the parasite (adapted from http://www.dpd.cdc.gov/dpdx/HTML/Toxoplasmosis.htm)



The life cycle can be explained by sexual phase and asexual phase (Figure 1-2). The asexual component consists of two distinct stages called tachyzoites and bradyzoites.

The tachyzoite stage defines the rapidly growing form of the parasite found during the acute phase of toxoplasmosis (Black and Boothroyd, 2000). Tachyzoites are approximately 6 x 2 μm and these infect and multiply within nucleated host cells (Weiss and Kim 2007), with a generation time of 6 to 8 hours, until lysis (after 6 or 7 generations by endodyogeny) releases progeny to infect neighbouring cells (Black and Boothroyd, 2000).

In the infected animal, tachyzoites differentiate into bradyzoites and form tissue cysts (Figure 1-2), which begin to appear 7 to 10 days post-infection (Black and Boothroyd, 2000). These bradyzoite bearing cysts are predominantly found in the central nervous system and muscle tissue, and they can stay for the normal life span of the host (Black and Boothroyd, 2000). This development of tissue cysts throughout the body of the host characterises the chronic stage of the asexual cycle, and ingestion of these through consumption of infected meat leads to rupture in the digestive tract and the release of the gastric peptidase resistant bradyzoites within (Blader and Saeij, 2009). These bradyzoites then infect the epithelium of the intestinal lumen, where they differentiate back to the rapidly dividing tachyzoite stage for rapid dissemination throughout the body via secondary lymphoid tissues, thereby completing the asexual cycle (Black and Boothroyd, 2000; Blader and Saeij, 2009).

Toxoplasma sexually reproduces only in felids (cats). In this definitive host, parasites infecting the intestinal epithelium undergo sexual reproduction producing oocysts which are shed in the faeces. Mature oocysts are approximately 12 μm in diameter and contain

eight infective sporozoites (Weiss and Kim, 2007). The sexual life cycle ends when oocysts are excreted by the cats in their faeces 3 to 10 days after infection (Black and Boothroyd, 2000). Unsporulated oocysts are shed in the cat's faeces for 1 to 2 weeks, in this time large numbers may be shed. These take between 1 and 5 days to sporulate in the environment and become infective, oocysts can then persist in the environment for several months and are extremely resistant to disinfectants, freezing, and drying (Alfonso, Thulliez and Gilot-Fromont, 2006).

In nature, intermediate hosts (including birds and rodents) become infected after ingesting soil, water or plant material contaminated with oocysts shed from the felid reservoirs. Cats themselves become infected after consuming intermediate hosts (e.g. birds and rodents) harbouring *T. gondii* tissue cysts, although they may also become infected directly following the ingestion of sporulated oocysts. Notably, animals bred for human consumption and wild game may also become infected after ingestion of sporulated oocysts from the environment leading to the presence of contaminating cysts in their tissues (dpd.cdc.gov/dpdx).

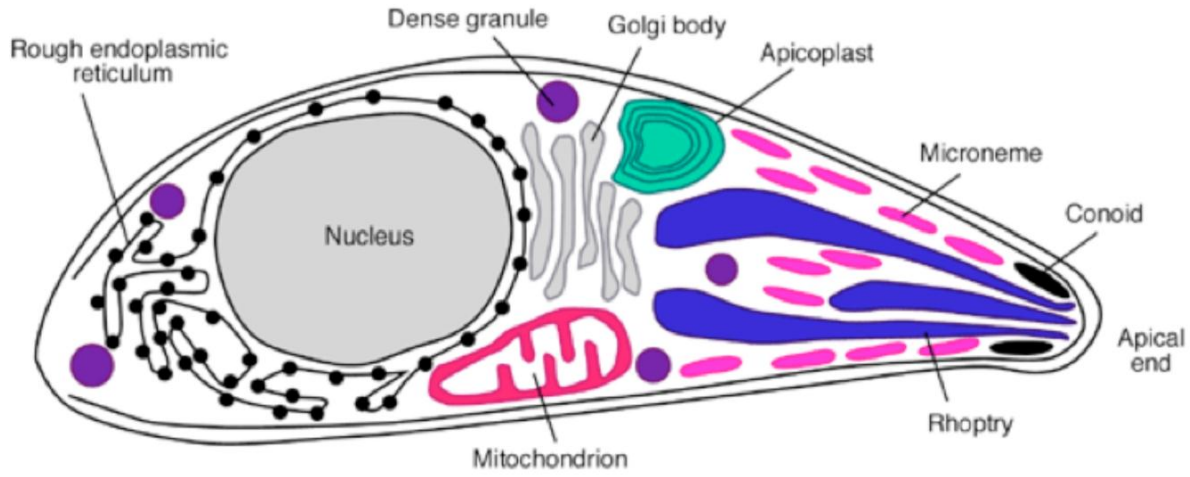
On ingestion cysts in contaminated meat disintegrate within the stomach and small intestine and the released sporozoites transform rapidly into tachyzoites (Alfonso, Thulliez and Gilot-Fromont, 2006). In cats, the multiplication and reproduction of *T. gondii* within the walls of the intestines leads to the shedding of oocysts as described. In intermediate hosts these tachyzoites disperse, as described above, primarily to muscle and nervous tissue, and to a lesser extent in lungs, liver and kidney, where they transform into bradyzoites and form tissue cysts which may persist for many years (Hill and Dubey, 2002). Humans enter this cycle either by consuming meat contaminated with cysts (raw or

undercooked) or through ingestion of oocyst contaminated food or water (Figure 1-2) (Hill and Dubey, 2002; Alfonso, Thulliez and Gilot-Fromont, 2006).

1.6 BIOLOGY OF *TOXOPLASMA GONDII*

As outlined above *Toxoplasma* exists in intermediate hosts, in two inter-convertible stages: bradyzoites and tachyzoites (Blader and Saeij, 2009). The crescent-shaped, disease causing tachyzoites possess a pellicle, subpellicular microtubules, a polar ring, a conoid, rhoptries, micronemes, mitochondria, a single stack of Golgi apparatus, ribosomes, rough surface endoplasmatic reticulum, micropores and a well-defined nucleus (Figure 1-3) (Petersen and Dubey, 2005; Joiner and Roos, 2002; de Graffenried, Ho and Warren, 2008).

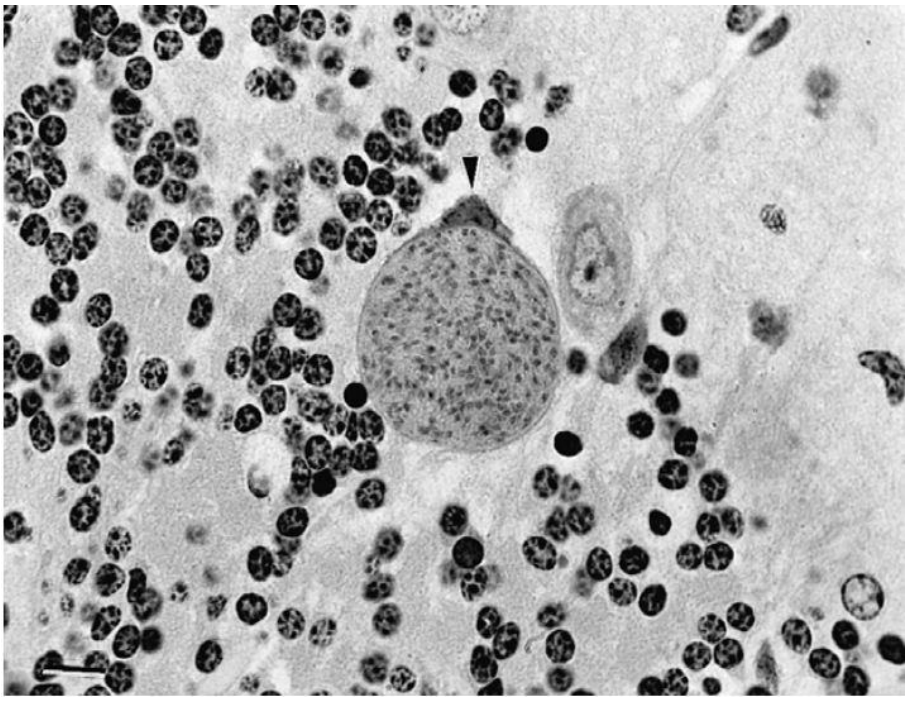
Figure 1-3: A schematic representation of ultra-structure of a *T. gondii* tachyzoite (adapted from http://journals.cambridge.org/fulltext_content/ERM/ERM3_01/S1462399401002204sup013.gif).



Subpellicular microtubules run longitudinally for almost the entire length of the parasite and give *Toxoplasma* its distinctive shape. The polar ring located anteriorly encircles the conoid, a cylindrical cone consisting of 6 to 8 fibrillar elements. Here the club-shaped rhoptries abut; these structures have a secretory function associated with host cell penetration (Petersen and Dubey, 2005). Close by are the grain-like micronemes also thought to participate in host cell invasion (Petersen and Dubey, 2005).

Tachyzoites activate a potent host immune response that eliminates most of the parasites. Some tachyzoites, however, escape destruction and convert into bradyzoites (Blader and Saeij, 2009). These forms then divide slowly by endodyogeny within tissue cysts (Figure 1-4) (Petersen and Dubey, 2005). A tissue cyst is a collection of bradyzoites surrounded by a well-defined host cell membrane, they develop in the host cell cytoplasm where its wall becomes tightly associated with the host endoplasmic reticulum (ER). Cysts are sized from 5 µm to 60 µm in the brain and up to 100 µm in other tissues (Petersen and Dubey, 2005). The formation of bradyzoites within cysts is required for the establishment of chronic infection. The process is driven by the host response to rapidly dividing tachyzoites and is marked by changes in the parasite expression profile (Blader and Saeij, 2009).

Figure 1-4: Intracellular *T. gondii* tissue cyst (arrow) in a section of mouse cerebellum (adapted Petersen and Dubey, 2005).



Notably, *T. gondii* can infect and replicate within any nucleated mammalian or avian cell. Within these the parasites replicate within a parasitophorous vacuole (PV) before egress and infection of neighbouring cells (Black and Boothroyd, 2000; Blader and Saeij, 2009). The PV is formed after invasion and is delineated by a parasitophorous vacuole membrane (PVM) which contains numerous intravacuolar tubules apparently connected to the parasite plasma membrane (Petersen and Dubey, 2005).

1.6.1 Host cell invasion process

Host cell invasion by *T. gondii* is a complex process with multiple, regulated stages. Firstly, the parasites attach to the host cell surface via a low affinity interaction largely mediated by GPI-anchored pathogen SAG proteins (surface antigens) (Blader and Saeij, 2009).

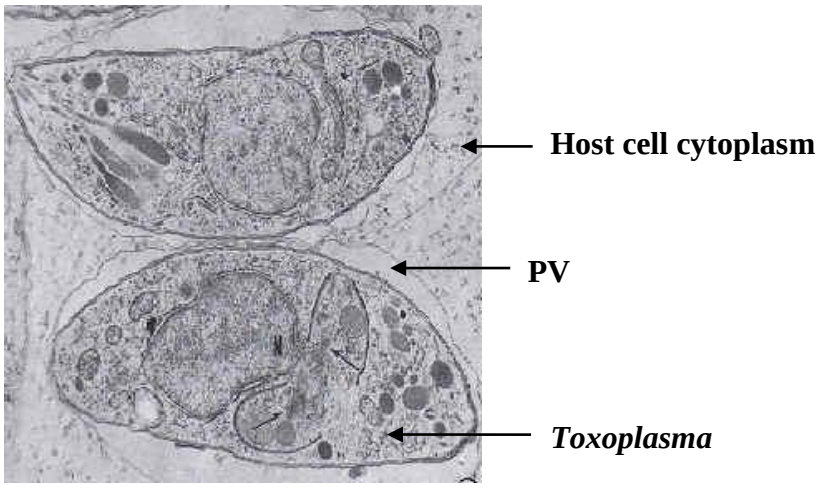
Subsequently, an unknown signal triggers an increase in cytosolic calcium stimulating microneme discharge (Blader and Saeij, 2009). More than 20 micronemal proteins have been identified and many are either transmembrane adhesins or adhesin accessory proteins (Blader and Saeij, 2009). Adhesin binding to host cells tightens the attachment of *T. gondii*, the parasite then utilises actin-myosin powered gliding motility for invasion (Blader and Saeij, 2009).

A second unknown trigger then stimulates the *T. gondii* to release the contents of the rhoptry bodies (Blader and Saeij, 2009). These rhoptry proteins function in the formation of a complex on the host cell plasma membrane known as the moving junction, this migrates down the entire length of the parasite as invasion proceeds (Blader and Saeij, 2009).

During penetration the PV is formed, largely from lipids derived from the host plasma membrane (Blader and Saeij, 2009). However, *T. gondii* regulated the protein composition of the PV by blocking the accumulation of most host transmembrane, but not GPI-linked, proteins (Blader and Saeij, 2009). The parasite forms a parasitophorous vacuole (PV) (Figure 1-5) immediately after invasion and resides within it while depending on the host for nutrients (Stedman *et al*., 2003).

Figure 1-5: Electron microscopy image of *Toxoplasma gondii* within parasitophorus vacuoles (PV) within the host cell cytoplasm.

(adapted from http://vmbmod10.msu.montana.edu/vmb/white-lab/photos.htm)



1.6.2 Interaction between the host and parasite

Within the PV the interactions between the parasite and host remain largely unknown. The PV does not fuse with host organelles, however it is closely associated with the host ER and mitochondrion (Martin *et al*., 2007). In addition, small soluble molecules (less than 1300-1900 Da) are able to cross PVM and enter the PV by diffusion (Schwab, Beckers and Joiner, 1994). The parasite also actively scavenges a variety of fatty acids and lipids from the host, some of which are further metabolised by the parasite (Coppens, 2006). Furthermore, recent evidence suggests that the relationship of *T. gondii* with its host cell extends beyond a simple parasitic relationship (i.e. scavenging and survival) and involves the manipulation of host metabolism (Weiss and Kim, 2007).

Importantly, *T. gondii* is unable to synthesize a number of essential nutrients *de novo* and therefore must be able to scavenge for these efficiently (Blader and Saeij, 2009). As mentioned above, small molecules (e.g. ions, amino acids, sugars, nucleosides, nucleotides and polyamines of less 1300-1900 Da) diffuse passively across the PVM. However, *T. gondii* must also scavenge the macromolecule cholesterol, and many studies have demonstrated that parasite replication is hindered in the absence of exogenous lipoprotein (Coppens *et al*., 2000).

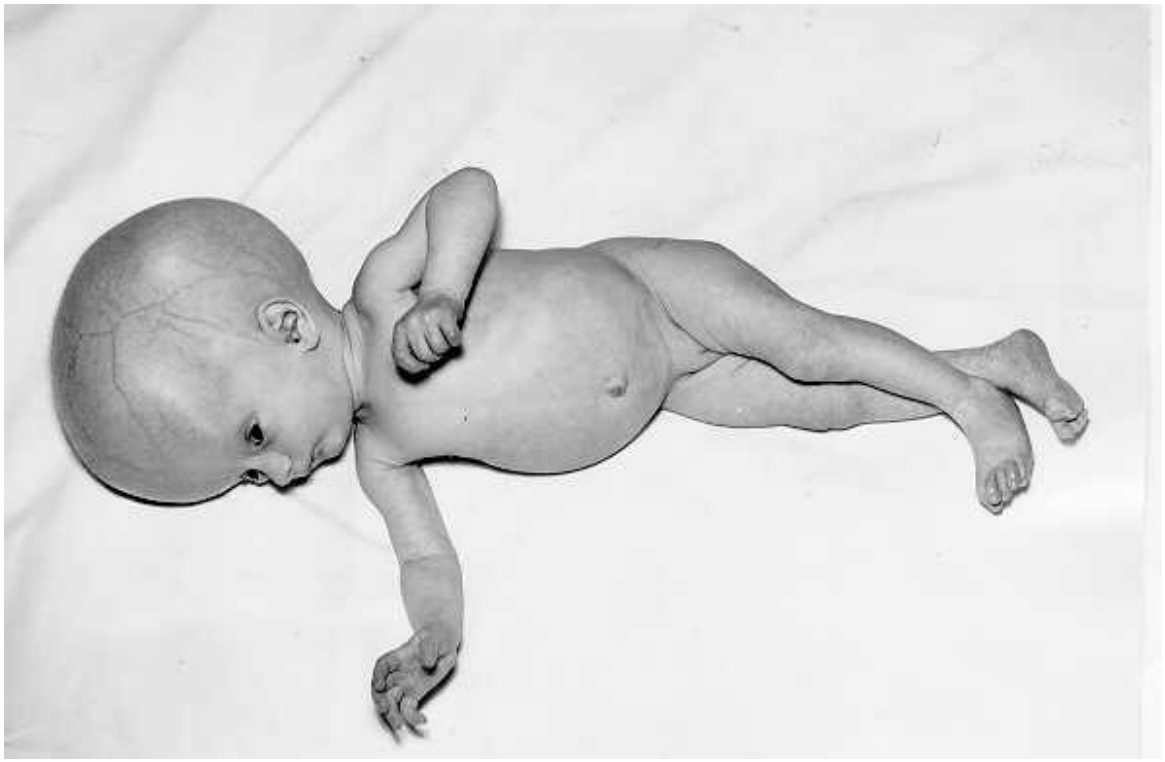
During invasion host cholesterol from the plasma membrane is incorporated into the PVM through a caveolae independent mechanism, and depleting plasma membrane cholesterol blocks parasite internalization (Bansall, Bhatti and Sehgal, 2005). However, post invasion *T. gondii* exploits host low density lipoprotein receptor-mediated endocytosis to acquire essential cholesterol (Bansall, Bhatti and Sehgal, 2005). Similarly, host-derived lipoic acid is scavenged, although the reasons for this are unclear given that lipoic acid is synthesized and used in the vestigal apicomplexan plastid organelle, the apicoplast (Crawford *et al*., 2006).

1.7 TOXOPLASMOSIS

1.7.1 Toxoplasmosis is caused by Toxoplasma gondii

Toxoplasmosis, the resulting disease from *T. gondii* infection, is a frequent cause of lethal cerebral pathology in immuno-compromised individuals and can also induce severe congenital anomalies (Figure 1-6) (Georgiev, 1994). In the past two decades, there has been a dramatic increase in the number of immuno-compromised individuals and thus a concomitant increase in severe toxoplasmosis (Black and Boothryod, 2000). Therefore, this disease represents an important concern in the AIDS community.

Figure 1-6: Congenital defects (hydrocephalus, liver enlargement and growth retardation) in infants (adapted from :http://gsbs.utmb.edu/microbook/ch084.htm)



The most damaging form of toxoplasmosis is congenital toxoplasmosis which results hydrocephalus, intracranial calcification, and chorioretinitis in foetus (Holland, 2003). Acute infections in pregnant women (and animals) can be vertically transmitted to the foetus and cause severe illness (toxoplasmosis.org). Notably, recent research suggested that congenital transmission may be much more important than previously thought in the spread of *T. gondii*, at least in animal populations (Hide *et al*., 2009).

Toxoplasmosis is the most widespread zoonosis in felines, and economically important agricultural and domestic animals such as dogs, pigs, cattle, poultry, sheep, goats and horses (Dubey and Jones, 2008). *T. gondii* has also been recorded in wild animal species, including game and captive zoo animals including endangered species and marine mammals (Dubey and Jones, 2008).

1.7.2 Geographical distribution of Toxoplasma in humans

T. gondii is a common parasite and toxoplasmosis is one of the most common infections throughout the world (dpd.cdc.gov/dpdx). Although the burden of this disease varies greatly from country to country, it remains a global public health problem which affects about one billion individuals. The recent discovery of its common infection in certain marine wildlife such as sea otters indicates contamination of seas with *T. gondii* oocysts washed from land (Dubey, 2008).

T. gondii varies in the severity of disease caused (benign to fatal) and the mode of transmission. Whilst investigating the role of genetic-variation in this it was demonstrated that whilst most *T. gondii* genotypes restricted to certain regions, some closely related types are found across continents. This indicated a recent radiation of a pandemic genotype (Lehmann *et al*., 2006). Evidence suggested that South American and Eurasian populations have evolved separately until recently, when ships populated by rats, mice, and cats provided *T. gondii* with unprecedented migration opportunities, probably during the transatlantic slave trade (Lehmann *et al*., 2006).

1.7.3 Disease burden

It is estimated 400-4,000 cases of congenital toxoplasmosis occur each year in the United States. These are primarily caused by the consumption of contaminated meat, making toxoplasmosis the third leading cause of food-borne deaths in USA (toxoplasmosis.org). A high prevalence of infection in France has been related to a preference for eating raw or undercooked meat, while a high prevalence in Central America has been related to the frequency of stray cats in a climate favouring survival of oocysts and soil exposure (toxoplasmosis.org).

1.7.4 Diagnosis

Toxoplasma infection can be diagnosed through the use of serological tests on blood or body fluids, amniocentesis (via PCR), isolation of the parasite and the characteristic microscopic appearance of tissues such as bronchoalveolar lavage material from immuno-compromised patients, or lymph node biopsy (toxoplasmosis.org; dpd.cdc.gov/dpdx).

1.7.5 Pathogenesis

Clinical disease is normally limited either to immuno-compromised individuals or to congenital disease resulting from an acute infection of the expectant mother. The severity of congenital infection depends upon the stage of pregnancy when the acute infection occurred, and spontaneous abortions or neurological disorders such as blindness and mental retardation may result (Black and Boothroyd, 2000). In immuno-compromised individuals (typically those with an untreated HIV infection) *Toxoplasma* is a frequent cause of intracerebral focal lesions resulting in toxoplasmic encephalitis, which can be fatal (Black and Boothroyd, 2000).

In immuno-competent humans, postnataly acquired toxoplasmosis is either unapparent, or accompanied by cervical lymphadenopathy with fever, joint pain, headache and tiredness (Kankova and Flegr, 2007). In addition, some congenitally infected infants are asymptomatic at birth, but can develop clinical signs in the later years, i.e. slower neurological and mental development and late chorioretinitis (Kankova and Flegr, 2007). Evidence suggests that most individuals with ocular toxoplasmosis were infected postnataly where ocular lesions may first develop many years after the infection. The risk of recurrent ocular disease appears to be greater during the first year after an episode of toxoplasmic retinochoroiditis than during subsequent years (Holland, 2003).

Within the host there is an apparent low rate of spontaneous reactivation whereby *Toxoplasma* bradyzoites differentiate back to tachyzoites (Black and Boothroyd, 2000). Normally, the immune response efficiently prevents the dissemination of these tachyzoites (Black and Boothroyd, 2000), but in immuno-compromised hosts this reactivation may go unchecked and / or more frequent. This lead to the suggestion that the parasites may actively detect a lowered immunity against them resulting in the massive and potentially fatal recrudescences observed (Black and Boothroyd, 2000).

1.7.6 Transmission and etiologic factors

T. gondii utilises both horizontal and vertical transmission (Hide, 2009). Generally, horizontal transmission occurs from individual to individual and is mediated by alternative hosts (cats and mice in the case of *T. gondii*). However, vertical transmission leads to transmission of *T. gondii* from one generation of host to the next by, for example, controlling the physiology of the host to promote transmission (Hide, 2009). Therefore, *T. gondii* must have evolved mechanisms to exploit both of these transmission routes to ensure its survival.

Transmission to humans can occur by three principal routes: i. ingestion of oocysts shed by cats; ii. ingestion of raw or inadequately cooked infected meat; and iii. congenital transmission (a newly infected pregnant woman passing the infection to her unborn foetus; Hide *et al*., 2009).

In addition to undercooked meat, drinking water and unwashed vegetables contaminated with oocysts may be an important source of infection (Hide *et al.*, 2009). Ingestion of cat faeces through hand-to-mouth contact following gardening, cleaning cat litter box and contact with children's sand pits may also lead to infection (Hill and Dubey, 2002;

Alfonso, Thulliez and Gilot-Fromont, 2006). In addition, blood transfusion and organ transplantation have been recognised as another source of transmission (dpd.cdc.gov/dpdx).

1.7.7 Recommendations for prevention

Hygiene remains the best preventive measure because currently there is no vaccine to prevent toxoplasmosis in humans (Dubey, 2008). Cooking meat to a safe temperature (i.e. one sufficient to kill *Toxoplasma*), peeling or thoroughly washing fruits and vegetables before eating, cleaning cooking surfaces and utensils after they have contacted raw meat, poultry, seafood, or unwashed fruits and vegetables are some of the preventive measures recommended (toxoplasmosis.org).

By recognising the risks many cases of congenital toxoplasmosis could be prevented. Specific measures that can be taken by women to decrease the infection risk during pregnancy include those mentioned above plus, for cat owners, avoiding changing the litter tray and / or limiting the risk of the cat becoming infected by not feeding it raw or undercooked meat and keeping it indoors to prevent it eating infected prey (toxoplasmosis.org).

1.7.8 Potential therapeutics for toxoplasmosis

Immuno-compromised or AIDS patients are given sulfadiazine and pyrimethamine (Kankova and Flegr, 2007). Folinic acid (leucovorin) is administered in all cases to reduce the bone marrow suppression caused by pyrimethamine. Spiramycin is given to pregnant women with acute toxoplasmosis (Kankova and Flegr, 2007). Although the current drug therapy will effectively kill the tachyzoite stage, such treatment does not remove the chronic bradyzoite stage, and thus long-term therapy is needed (Black and Boothroyd, 2000). A live tachyzoite vaccine, Toxovax is the only commercially available vaccine

worldwide to protect against congenital toxoplasmosis in ewes (Innes *et al*., 2009 and Buxton *et al*., 2007). This suggests that it may also reduce or prevent tissue cyst development in muscle. However, the question of whether the vaccine could be used to make meat safer for human consumption is still unanswered (Buxton *et al*., 2007).

While chemotherapy is the major control strategy for these parasites, drugs to treat these parasites are limited by their inability to eliminate the infection, emerging resistance and poor patient tolerance (Derouin, 2005). Since resistance is often due to mutations within drug target sites, there is a genuine need not only for new drugs, but also for the identification of novel chemotherapeutic targets (Aspinal *et al*., 2002).

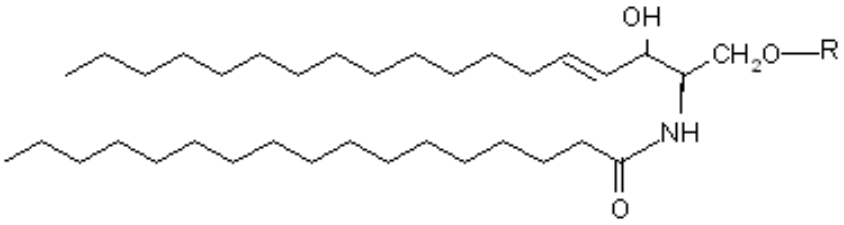
1.8 LIPIDOMICS AND DRUG TARGETS

Investigations of lipids and their biosynthesis can increase understanding of the lipid-regulated physiopathological events and perhaps indicate new therapeutic strategies (Singh and Del Poeta, 2011). Identifying cellular processes that are unique and essential to the parasite is therefore a crucial step towards defining appropriate drug targets. Recent achievements in this field are promising and suggest that the elucidation of lipid pathways will provide new opportunities for designing potent anti-parasitic strategies (Denny *et al*., 2006). One essential apicomplexan metabolic process that may be exploited for the development of anti-parasitic drugs is the fatty acid biosynthetic pathway (Goodman and McFadden, 2007). Another potential pathway for intervention is sphingolipid metabolism; here the parasites possess several novel enzymes that could be exploited for the development of new drugs for toxoplasmosis (Sonda *et al*., 2005).

1.8.1 Structure and diversity of sphingolipids

Sphingolipids were first discovered by J. L.W. Thudichum in 1876 and are ubiquitous constituents of membranes in eukaryotic organisms. They fall into 3 major categories: ceramides (the simplest or 'base' sphingolipid), phosphosphingolipids (e.g. sphingomyelin) and glycosphingolipids. They are a structurally diverse family of lipids consisting of a sphingosine backbone with a long chain fatty acid with, in the case of complex sphingolipids, a polar head group (Figure 1-7) (Landoni *et al*., 2007; Koeller and Heise, 2011).

Figure 1-7: Chemical structure of a complex sphingolipid representing the sphingosine backbone and the long chain fatty acid. R is the head group, H in the case of ceramide.



The polar head groups of complex sphingolipids show a striking structural diversity among phylogenetically separate organisms (Hanada, 2003). In mammalian cells, choline phosphoceramide (sphingomyelin) accounts for approximately 10% of membrane phospholipids, while various lower animal cells have ethanolamine phosphoceramide and its N-mono or dimethyl derivatives in place of sphingomyelin (Hanada, 2003). In contrast, plants and fungi produce phosphoinositol-containing sphingolipids (inositol phosphoryl ceramide) (Hanada, 2003). With respect to glycosphingolipids, numerous variations in the glycosyl head structure exist even within in the same organism (Hanada, 2003).

1.8.2 Sphingolipid functions and roles in pathogenicity

For a long time these species were considered to primarily play a structural role in membrane formation (Bartke and Hannun, 2008). However, intensive studies of sphingolipid metabolism and function has revealed ceramide, sphingosine, sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), as bioactive molecules playing roles from regulation of signal transduction pathways, through direction of protein sorting to the mediation of cell-to-cell interactions and recognition (Bartke and Hannun, 2008; Singh and Del Poeta, 2011).

Sphingolipids also play important roles through their abundance and involvement in membrane microdomain or raft formation, as well as serving as the lipid anchor for many of these molecules which function as in signal transduction and protein sorting (Zhang *et al*., 2010; Bartke and Hannun, 2008). In these ways sphingolipids and their metabolites modulate proliferation, differentiation, growth suppression, apoptosis and also, pathogen infection and host defense (Landoni *et al*., 2007; Hullin-Mastuda and Kobayashi, 2007). For example, microbial sphingolipids and their metabolizing enzymes have a pivotal role in the regulation of fungal pathogenicity, notably in *Cryptococcus neoformans*. In this case sphingolipids mediate cellular processes such as cell growth, proliferation, apoptosis and senescence (Landoni *et al*., 2007).

In addition sphingolipid synthesis and signaling renders the pathogenic fungi hypervirulent by making them resistant environmental stresses and killing by the host immune responses (Singh and Del Poeta, 2011). With respect to protozoan pathogens, the interactions of parasite sphingolipid metabolism with that of the host may be important in parasite survival and / or host defence (Zhang *et al*., 2010).

1.8.3 Ceramide

The simplest sphingolipid, ceramide is a precursor of complex sphingolipids and a major player in cell signalling (Block *et al*., 2007). This lipid is a key signalling molecule in processes including apoptosis, growth arrest, senescence, differentiation, the immune response and cell cycle arrest (Baier and Barrantes, 2007).

Cellular ceramide can be generated by hydrolysis of sphingomyelin via an acid or neutral sphingomyelinase (Baier and Barrantes, 2007; Holthuis *et al*., 2001). Another important source of ceramide is from the breakdown of glycosphingolipids through the removal of their terminal hydrophilic groups by a series of specific hydrolases (Hannun and Obeid, 2008). In all cases the released ceramide can be either recycled into sphingolipid synthesis or degraded to sphingosine by ceramidases (Merrill *et al*., 2002). Sphingosine can then be phosphorylated by sphingosine kinases to generate sphingosine-1-phosphate (S1P), an intracellular second messenger and extracellular ligand for specific receptors (Holthuis *et al*., 2001).

Ceramide can also be synthesized *de novo* in the ER through the action of ceramide synthase, subsequently this lipid can be translocated to the Golgi apparatus where it is modified to complex phospho- or glyco-sphingolipid (Cremetsi, Goni and Kolesnick, 2002). It has been suggested that transport of proteins through the secretory pathway is coupled to sphingolipid biosynthesis (Holthuis *et al*., 2001).

1.8.4 De novo synthesis of sphingolipids

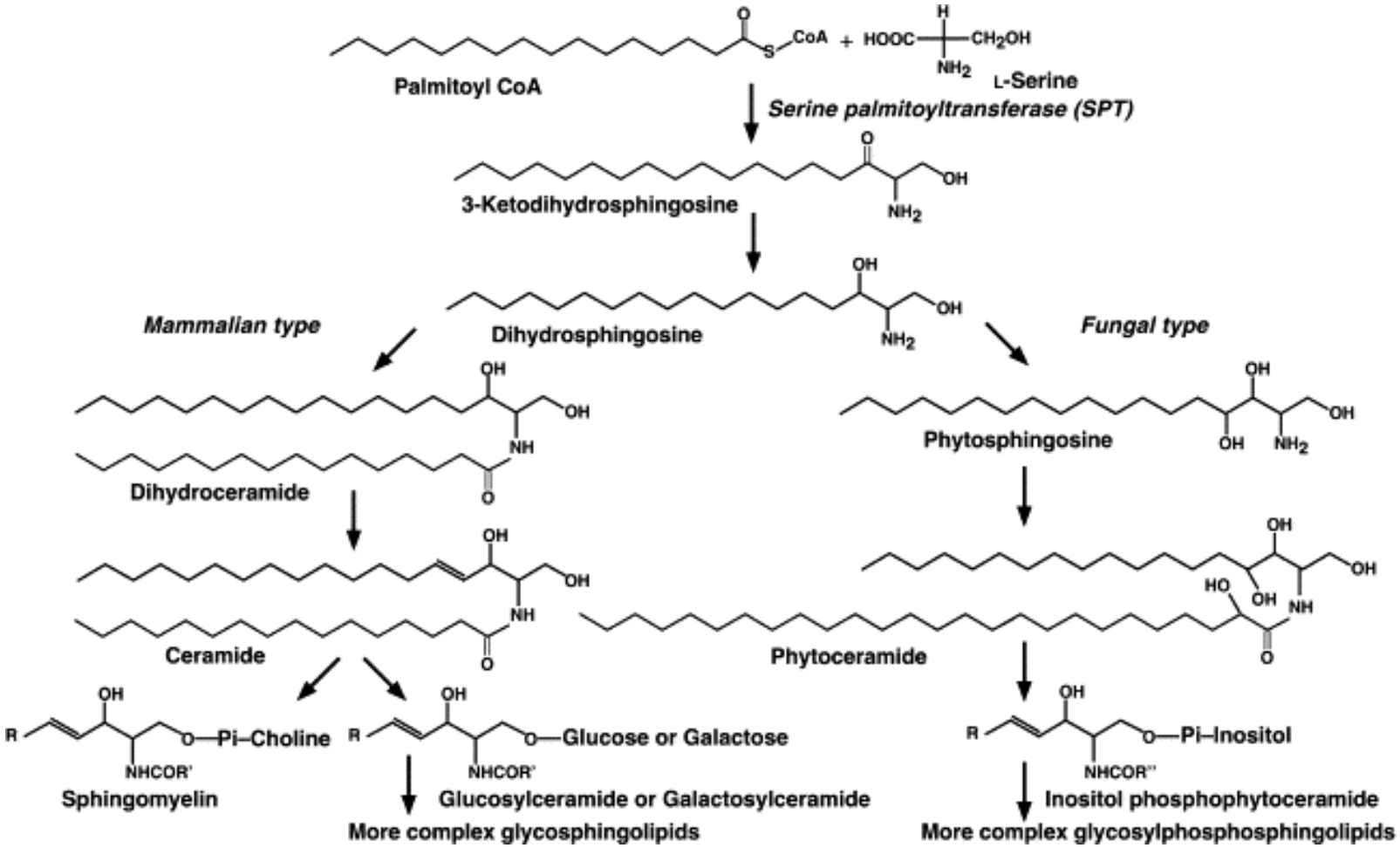
Despite the diversity evident in eukaryotic sphingolipids, serine palmitoyltransferase (SPT) is the enzyme ubiquitously responsible for the initial synthetic step (Figure 1-8). This limiting step is the condensation of L-serine and palmitoyl CoA to produce 3-ketodihydrosphingosine (KDS) (Hanada, 2003; Merrill, 2002). In mammals and yeast at least two gene products (termed LCB 1 and LCB 2, or sometimes SPT 1 and SPT 2) responsible for this activity have been identified (Cheng *et al*., 2010).

In the second step, KDS is reduced to form dihydrosphingosine (Figure 1-8). In mammalian cells this is *N*-acylated by ceramide synthase to form dihydroceramide, which is then de-saturated to form ceramide, which is primarily used for the synthesis of sphingomyelin (Figure 1-8 SM; Hanada, 2003). SM synthase transfers a phosphocholine head group from phosphatidylcholine (PC), generating SM and also diacylglycerol (DAG) and water as by-products (Hannun and Obeid, 2008). Ceramide can also be phosphorylated by ceramide kinase, which in turn can be recycled by a ceramide-1-phosphatase (C1P); or glycosylated by glucosyl or galactosyl ceramide synthases (Figure 1-8) (Hannun and Obeid, 2008).

In contrast, in fungal (and plant) cells, dihydrosphingosine is hydroxylated to generate phytosphingosine, which is *N*-acylated to produce hydroxyceramide (Figure 1-8 phytoceramide; Hanada, 2003). The prevalent acyl chain of fungal phytoceramide is C26 hydroxy fatty acid, whereas typical acyl chains of mammalian ceramide are saturated C16 and C24 fatty acids (Hanada, 2003). In both plant and fungal cells phytoceramide is converted to inositol phosphorylceramide (IPC), a species absent from mammalian cells, whose formation is catalysed by IPC synthase (Figure 1-8; Hanada, 2003). This enzyme

catalyses the transfer of phosphoinositol from phosphatidylinositol to phytoceramide to produce IPC which is then used to produce more complex phosphosphingolipids, such as glycosylated phosphosphingolipids in plants and mannose-inositol phosphoryl ceramide in yeast (Merrill and Sandhoff, 2002).

Figure 1-8: Sphingolipid biosynthetic pathway of mammalian and fungal cells (adapted from Hanada, 2003)

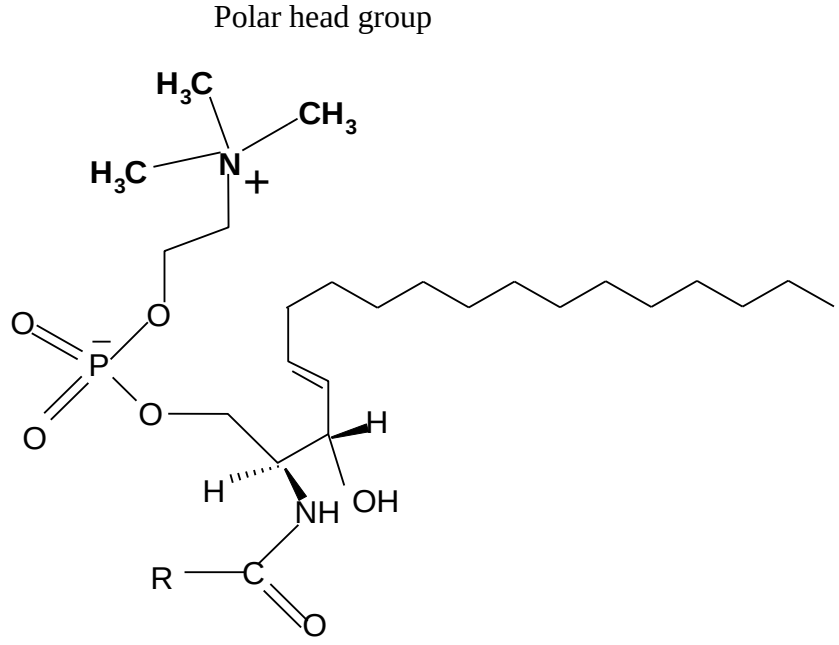


Therefore, there is evolutionary divergence in the pathway, with the final products of the sphingolipid biosynthetic pathways being different in mammals, fungi and plants (Figure 1-8). Thus, certain steps of this pathway are potential targets for chemotherapy against fungal pathogens (Koeller and Heise, 2011).

1.8.5 Sphingomyelin (SM) and SM synthase

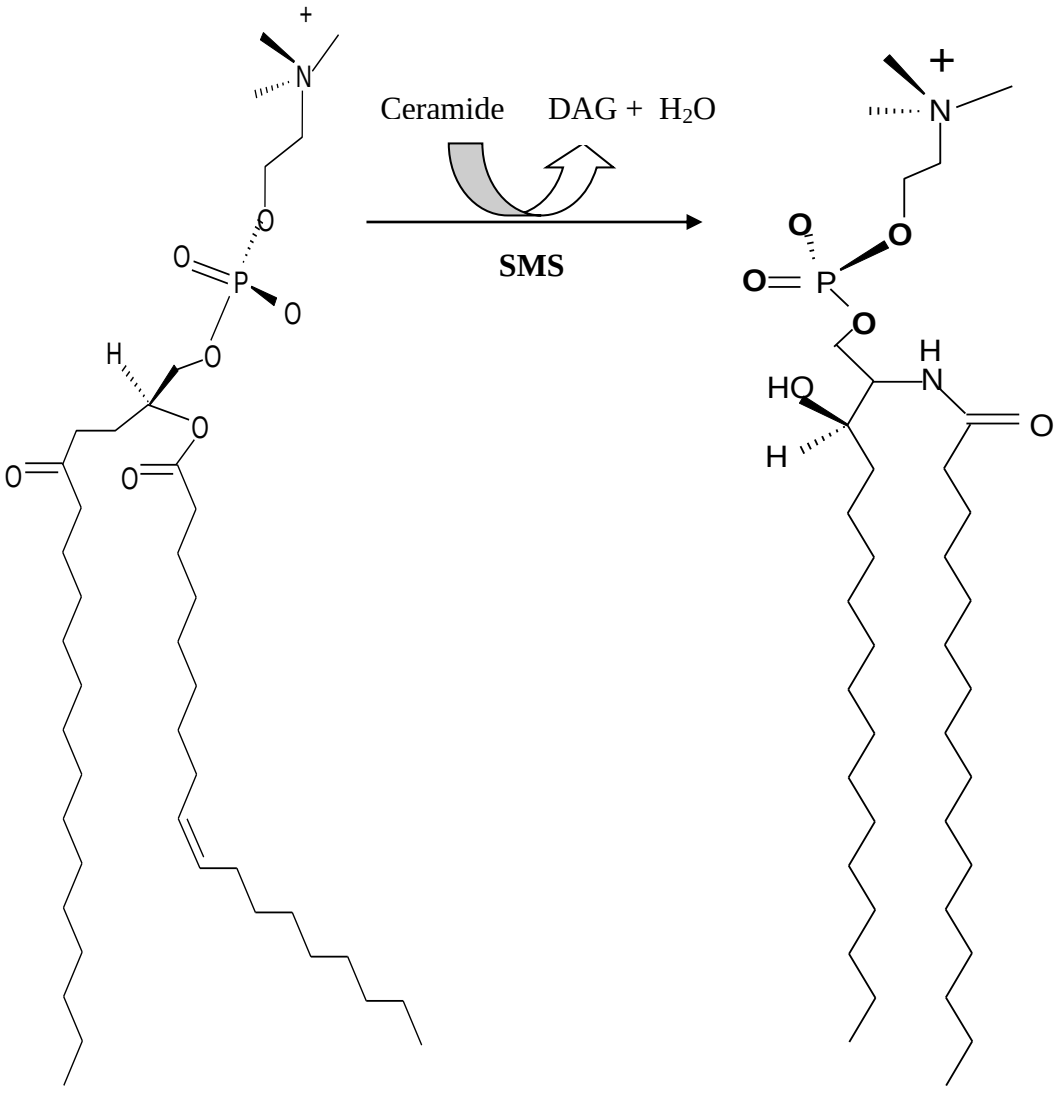
Sphingomyelin (SM) is the primary phosphosphingolipid of animal cell membranes and mammalian cells (Koeller and Heise, 2011). Plants, fungi and yeast do not produce SM (Rosenwald, Machamer and Pagano, 1992). This lipid is composed of phosphocholine and ceramide (Figure 1-9).

Figure 1-9: Chemical structure of sphingomyelin. Sphingomyelin is composed of a ceramide backbone with a phosphocholine head group attached to the C-1 free alcohol group of ceramide. The fatty acid (R) is attached to the second carbon of sphingosine via an amide linkage.



This molecule is formed by the transfer of the phosphocholine head group from phosphatidylcholine (PC) to ceramide (Figure 1-10) (Koeller and Heise, 2011). This process is mediated by SM synthase (SMS) in the lumen of the Golgi apparatus and on the cell surface (Rosenwald, Machamer and Pagano, 1992). SMS also catalyses the reverse reaction as it is in equilibrium with substrates (PC and ceramide) and products (SM and diacylglycerol) (Melendez, 2008).

Figure 1-10: A schematic representation of chemical reaction in the formation of sphingomyelin from phosphatidylcholine and ceramide. In this reaction, phosphocholine head group of phosphatidylcholine is transferred on to ceramide, resulting in sphingomyelin, diacylglycerol (DAG) and water. This reaction is catalysed by sphingomyelin synthase (SMS).

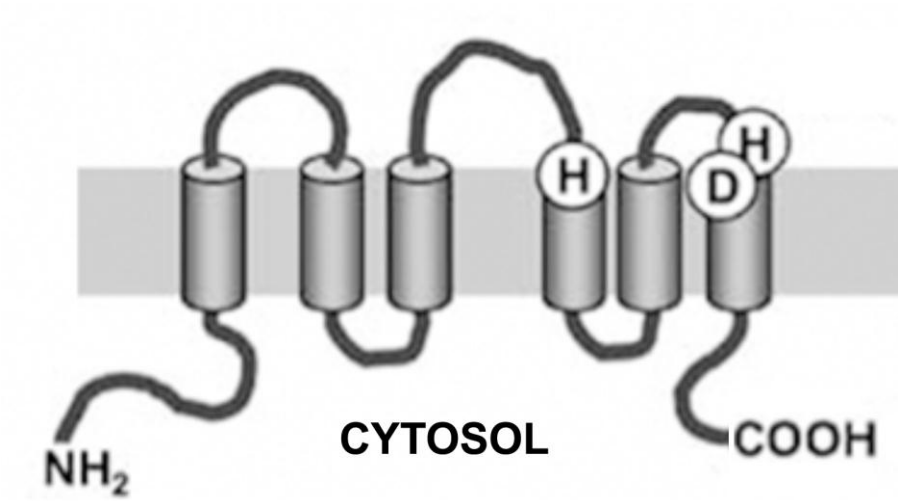


Phosphatidylcholine **Sphingomyelin**

In the human genome there are two genes encoding sphingomyelin synthase denoted as *Hs* SMS 1, *Hs* SMS 2 whose products are localized in Golgi and on the plasma membrane respectively (Huitema *et al*., 2004). It is predicted that other animal genomes also contain at least 2 SMS genes (Huitema *et al*., 2004; Koeller and Heise, 2011).

The catalytic mechanism employed by the SM synthase requires a catalytic triad consisting of two histidines and an aspartate residue. The transmembrane arrangement of the enzyme means that this triad is presented towards the lumen of the Golgi in the case of *Hs* SMS 1 and the extracellular environment in the case of *Hs* SMS 2 (Figure 1-11) (Huitema *et al*., 2004). In this the SM synthases resemble the lipophosphotransferases (LPPs). In the case of SMS the choline phosphate residue is proposed to be transferred from phosphatidylcholine to an activated histidine, this is then subjected to nucleophilic attack by the oxygen of the ceramide primary hydroxyl group, resulting in transfer to the sphingoid base (Figure 1-11; Huitema *et al*, 2004). This triad lies within domains 3 and 4 (D3 and D4) of the SMS proteins, 2 other domains of unknown function are also conserved (D1 and D2; Huitema *et al*., 2004).

Figure 1-11: Membrane topology of *Hs* SMS 1/2 proteins showing the 6 transmembrane domains and the conserved catalytic triad incorporating two histidines (H) and one aspartate (D) residues in domains 3 and 4 (adapted from Huitema *et al*., 2004).



1.8.6 Inositol phosphorylceramide (IPC) and IPC synthase

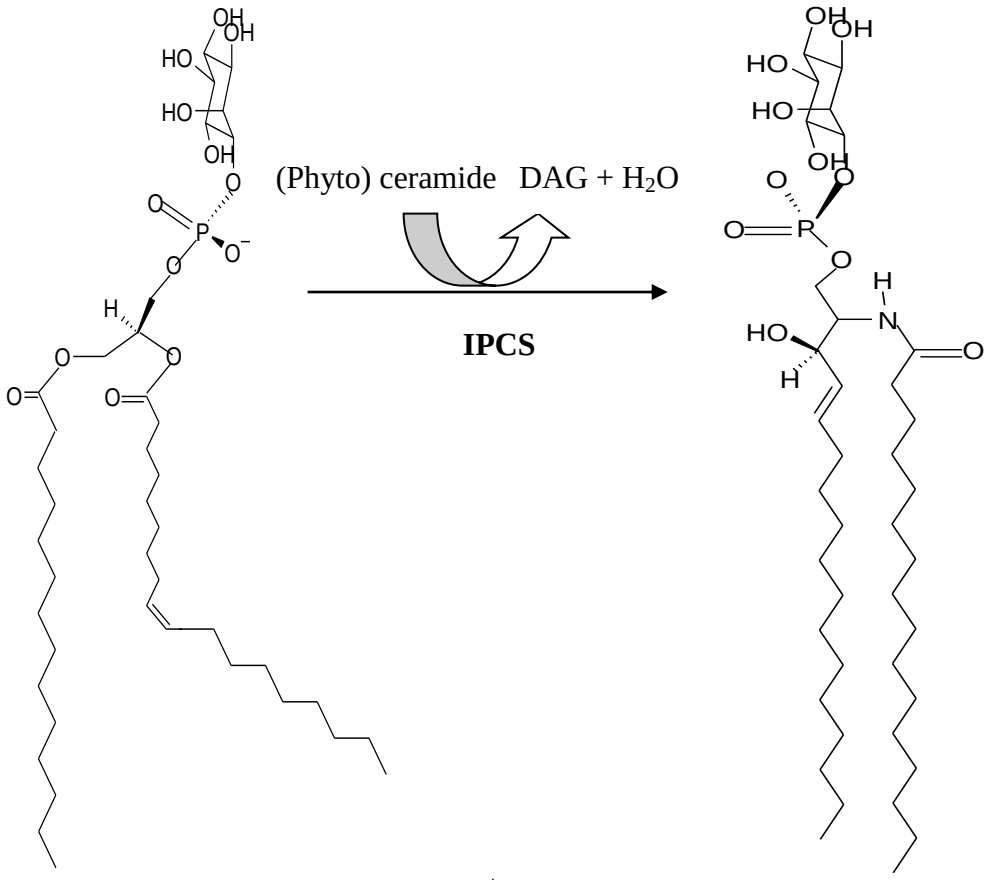
Plants, fungi, and some protozoa synthesize inositol phosphorylceramide (IPC) rather than SM as their primary phosphosphingolipid (Koeller and Heise, 2011). Inositol phosphorylceramide (IPC) synthase is an enzyme common to both fungi (where it is known as the AUR1, Aureobasidin resistance, protein) and plants (Sugimoto, Sakoh and Yamada, 2004). Its product, like SM in mammalian cells, is known to be a major component of the plasma membrane. However, yeast, plants and certain protozoa also incorporate IPC into GPI-anchors for plasma membrane proteins (Koeller and Heise, 2011).

IPC synthase (AUR1) catalyses the transfer of inositol phosphate (phosphoinositol head group) from phosphatidylinositol (PI) to C-1 hydroxy of ceramide to produce IPC (Figure 1-12) (Koeller and Heise, 2011; Sugimoto, Sakoh and Yamada, 2004; Denny *et al*., 2006). AUR1 is localized to the Golgi of yeasts and possess the same catalytic triad as the animal SM synthases (Levine *et al*., 2000), however it lacks regions corresponding to D1 and 2

(Huitema *et al*., 2000). Notably, mutation of His-294, part of catalytic triad in the yeast AUR1p, results in nonviable haploid cells (Levine *et al*., 2000). Recently an accessory protein involved in IPC synthase activity (KEI1p) has been identified (Sato *et al*., 2009).

AUR1 has been shown to have a role in hyphae formation in *Aspergillus nidulans* though regulation actin cytoskeleton (Singh and Del Poeta, 2011). In addition, AUR1 regulates the level of (phyto) ceramide (Figure 1-8; the acceptor substrate of the AUR1 reaction) and this is a substrate for ceramidases for the production of the apoptotic inducers phyto- and dihydrosphingosine (Cerbón *et al*., 2005; Singh and Del Poeta, 2011). This enzyme also controls the levels of diacylglycerol (DAG; Figure 1-8) which acts as a mitogenic signal (Cerbón *et al*., 2005). Thus AUR1, and by extension SM synthase, regulates the balance between apoptosis (cell death) and mitogenesis (cell growth) (Mina *et al*., 2011).

Figure 1-12: A schematic representation of chemical reaction in the formation of inositol phosphorylceramide from phosphatidylinositol and ceramide. In this reaction, phospholinositol head group of phosphatidylinositol is transferred on to ceramide, resulting in inositol phosphorylceramide, diacylglycerol (DAG) and water. This reaction is catalysed by inositol phosphorylceramide synthase (IPCS).



Phosphatidyl inositol **Inositol phosphorylceramide**

The *Saccharomyces cerivisiae* AUR1 protein is, therefore, essential (Nagiec *et al*., 1997). Given that IPC synthases have no functional mammalian equivalent, the enzyme is well established as a target for antifungals (Georgopapadakou, 2000) and inhibition of this essential biosynthetic step leads to the accumulation of phytoceramide in growing cells and cell death (Fischl *et al*., 1986). The cyclic depsipeptide aureobasidin A (Ab A) is the ‘gold

standard' AUR1 inhibitor and is widely used as a selectable marker for various plasmids (e.g. Clontech, Matchmaker Gold).

1.9 PROTOZOAN AND PLANT SPHINGOLIPIDS AND THEIR SYNTHESIS

Notably, many protozoan parasites also synthesize IPC as their primary phosphosphingolipid (Smith and Denny, 2004). For example, *T. gondii* has been indicated to synthesize IPC and possess an IPC synthase activity that can be inhibited by Ab A (Sonda *et al*., 2005). IPC is also the predominant phosphosphingolipid in the related kinetoplastid *Leishmania* species (Denny *et al*., 2004). The first protozoan IPC synthase to be identified and characterised was from *L. major* (Denny *et al*., 2006). Notably, the *Leishmania* enzyme *Lmj* IPCS, and its orthologues in the kinetoplastid pathogens, possess a conserved catalytic triad in the same conformation as the yeast and mammalian enzymes. However, unlike AUR1 they also have the D1 and D2 regions found in the animal SM synthases (Zhang, Bangs and Beverley, 2010).

This work has led to much interest as to whether IPC synthase could be a novel drug target for leishmaniasis, a neglected tropical disease affecting 12 million of the world's poorest people (Mina *et al*., 2010 b). The same argument could be made for the functionally equivalent enzymes in other kinetoplastid pathogens such as *T. cruzi* (Chaga's disease) and *T. brucei* (the cause of African Sleeping Sickness) or, for example, *T. gondii.* However, at the beginning of this work these enzymes remained undiscovered, as did the plant IPC synthase.

1.10 THE APICOMPLEXA

1.10.1 Toxoplasma gondii

De novo synthesis of ceramide, glycosphingolipids and SM in *T. gondii* has been demonstrated by metabolic labelling with tritiated serine and galactose, followed by analyses using thin layer chromatography (Azzouz *et al*., 2002). The incorporation of serine into sphingolipids indicated the presence of an active serine palmitoyltransferase (SPT) and the two well-documented SPT inhibitor, L-cycloserine, strongly inhibited the synthesis of these sphingolipids (Azzouz *et al*., 2002). In addition, lipidomic analyses using mass spectrometry has shown ethanolamine phosphorylceramide (EPC) to be relatively abundant in *T. gondii* compared to host cells (Welti *et al*., 2007). These studies did not identify IPC, however Ab A treatment inhibits *T. gondii* replication irreversibly and induces morphological changes in the parasite cell shape and integrity, with increased cell vacuolization (Sonda *et al*., 2005). In addition, analyses of parasite lipids revealed an inositol-containing species that was resistant to alkaline treatment which suggested the presence of IPC (Sonda *et al*., 2005). However, it is not yet clear whether IPC synthesis is required for parasite replication and more definitive characterizations of *T. gondii* sphingolipids and the enzymes involved in their synthesis is of paramount importance.

1.10.2 Plasmodium falciparum

Plasmodium species are, like *T. gondii*, apicomplexan parasites. They are the causative agents of malaria residing in mammalian red blood cells, hepatocytes, or in mosquito midgut epithelial cells. Biosynthesis of SM and glycosphingolipids by *P. falciparum* intra-erythrocytic stage of parasite has been demonstrated using radio-labelled and fluorescent ceramides (Haldar *et al*., 1991; Gerold and Schwarz, 2001). More recently, a putative SM synthase has been identified (Huitema *et al*., 2004). An active glucosylceramide synthase has also been identified and sulphated glycosphingolipids are evident in *P. falciparum*

however, there is no evidence for the presence of IPC or other inositol-sphingolipids (Zhang, Bangs and Beverley, 2010).

P. falciparum sphingolipid biosynthesis and parasite development can be inhibited with *threo*-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) or its *threo*-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) derivative, both of which are potent inhibitors of glucosylceramide synthase and sphingomyelin synthase (Couto *et al*., 2004). Although, the precise mechanism of action of PPMP and PDMP with respect to *P. falciparum* is unknown their potent activity *in vitro* suggests that sphingolipid biosynthesis could provide a target for new antimalarials.

1.11 THE KINETOPLASTIDAE

The Kinetoplastidae cause a range of neglected human diseases (WHO.int), including the leishmaniases (a broad spectrum of infections caused by *Leishmania* species), Chaga's disease (*Trypanosoma cruzi*) and African sleeping sickness (*Trypanosoma brucei*) (Stuart *et al*., 2008). Together these affect 20-30 million people, largely in the developing world. Currently there are no effective vaccines and many of the drugs available to treat these diseases are expensive, difficult to administer or toxic, compromised by low efficacy and resistance is rising (Cobb and Denny, 2010). Therefore the discovery of new anti-kinetoplastid therapeutic targets and compounds is of great importance. Recently, functional orthologues of the AUR1p have been identified in the Kinetoplastida and these may represent novel drug targets (Denny *et al*., 2006; Koeller and Heise, 2011).

1.11.1 Trypanosoma species

The trypanosome species cause two major and distinct human diseases: African Trypanosomiasis (Sleeping Sickness, *T. brucei spp*); and South American Trypanosomiasis or (Chaga's Disease, *T. cruzi*) (Stuart *et al*., 2008). However, there are marked differences within the genus with respect to life cycle, biochemistry, geographical distribution, and disease pathology. This is reflected in significant differences in sphingolipid composition and metabolism between *T. brucei* and *T. cruzi* (Zhang, Bangs and Beverley, 2010).

1.11.2 Trypanosoma cruzi

Trypanosoma cruzi (South American trypanosome) is the causative agent of human Chaga's disease, a neglected tropical disease affecting an estimated 10 million people mostly in Latin America (WHO.int). *T. cruzi* has a complex lifecycle complex with multiple morphological forms, including an extracellular phase in the triatomine insect ('kissing bug') vector and an intracellular stage inside the vertebrate host (Koeller and Heise, 2011). These lifecycle stages possess neutral glycosphingolipids such as gluco-, galacto-, and lactosylceramides (Koeller and Heise, 2011). In addition, they depend upon a variety of parasite synthesized GPI-anchored surface glycoconjugates, and therefore the biosynthetic pathways of GPI anchor biosynthesis have been proposed as attractive targets for new therapies for Chaga's disease (Koeller and Heise, 2011).

Unlike in *Leishmania* species and *T. brucei*, many of these anchors incorporate IPC (Sugimoto, Sakoh and Yamada, 2004). However, like *Leishmania* species *T. cruzi* are known to synthesize this non-mammalian phosphosphingolipid using an IPC synthase (Denny *et al*., 2006; Figueiredo *et al*., 2005). SM has also been reported in insect stage epimastigotes (Quinones *et al*., 2004), however radio-labeling with [^{3}H] palmitate only showed the presence of synthesized IPC in epimastigotes and mammalian stage

amastigotes (Salto *et al*., 2003). Unlike yeast and plant IPC in which phytoceramide predominates, the *T. cruzi* species contains a mixture of dihydroceramide and ceramide (Berello *et al*., 1995), in this it resembles *L. major* (Hsu *et al*., 2007). But like fungi and plants, and unlike *Leishmania* species, *T. cruzi* IPC can be modified *in vivo* by glycosyl groups to form free glycoinositolphospholipids (GIPLs) which are present on the parasite cell surface in all lifecycle stages (Salto *et al*., 2003).

Ab A has been proposed to inhibit the *T. cruzi* IPC synthase, with 10-20 μM impeding the differentiation of insect stage forms to amastigotes *in vitro* (Salto *et al*., 2003). However, it had no effect on the growth of epimastigotes at high concentrations, and was completely ineffective against IPC synthase in a direct enzymatic assay with epimastigote membranes (Figueiredo *et al*., 2005). Similarly, the *L. major* IPC synthase has been demonstrated to be Ab A resistant (Denny *et al*., 2006). Therefore, the inhibition of *T. cruzi* differentiation is likely to have been due to off-target effects (Figueiredo *et al*., 2005). However, the *T. cruzi* IPC synthase remains a promising drug target (Koeller and Heise, 2011).

1.11.3 Trypanosoma brucei

Trypanosoma brucei species are the causative agents of another neglected tropical disease, human African trypanosomiasis (HAT or African Sleeping Sickness) which affects many of the most under-developed regions of Africa. It kills in excess of 50,000 people annually and many of the drugs available are expensive and have severe toxicity (Barrett and Burchmore, 2003). Like *Leishmania* species and *T. cruzi*, *T. brucei* is an insect vector borne kinetoplastid, with the Tstse fly harbouring insect stage procyclic forms (PCFs) before differentiation to bloodstream forms (BSFs) on inoculation into a mammalian host. In both stages *T. brucei* is an extracellular parasites never residing within a host cell (Simarro, Jannin and Cattand, 2008).

Analyses of extracts from PCFs and BSFs by mass spectroscopy has confirmed the presence of both SM and IPC, as well EPC (Guther *et al*., 2006; Sutterwala *et al*., 2008). In addition, neutral glycosphingolipids have been identified in BSFs (Uemura *et al*., 2006). Interestingly, whilst IPC is dominant in PCF *T. brucei*, SM is present in both lifecycle stages and EPC is only found in BSFs (Sutterwala *et al*., 2008). Like *T. cruzi*, the phosphosphingolipids contain a mixture of dihydroceramide and ceramide, however for unknown reasons the first is favoured in PCFs and the other in BSFs (Sutterwala *et al*., 2008).

Inhibition of the *T. brucei* SPT, either pharmacologically (Sutterwala *et al*., 2007) or genetically (Fridberg, 2008), is lethal but can be rescued with 3-ketodihydrosphingosine the product of SPT (Sutterwala *et al*., 2007). This contrasts with *Leishmania* species where loss of SPT is tolerated (Denny *et al*., 2004; Zhang *et al*., 2003; Mullin *et al*., 2001). This indicated that sphingolipid biosynthesis is essential for *T. brucei* and therefore a drug target. Like *T. cruzi*, *T. brucei* encodes orthologues of the *L. major* IPC synthase (Denny *et al*., 2006). These 4 isoforms may represent promising drug targets but at the start of this project they were uncharacterised.

1.12 *ARABIDOPSIS THALIANA*

In the model plant *Arabidopsis thaliana*, the major sphingolipid types are glycosylinositolphosphoceramides, glucosylceramides, hydroxyceramides (phytoceramides) and ceramides (Markham and Jaworski, 2007). Several enzymes involved in plant sphingolipid metabolism have characterized (Pata, Hannun and Nq, 2010; Dunn *et al*., 2004). However, at the start of this project the key IPC synthase remained unidentified despite the availability of the complete genome sequence, although the enzyme activity has been measured in bean microsomal extracts and shown to be acutely sensitive to Ab A (Bromley *et al*., 2003).

CHAPTER 02

OBJECTIVES OF THE STUDY

2 OBJECTIVES OF THE STUDY

The apicomplexan protozoan parasite *Plasmodium falciparum* encodes two sequence orthologues of the human sphingomyelin synthases (*Pf* SMS 1 and 2; Huitema *et al*., 2004) and is known to synthesize SM (Haldar *et al*., 1991; Gerold and Schwarz, 2001). However, the *Pf* SMS proteins have not been functionally characterised. Furthermore, orthologues in other apicomplexans were yet to be identified. For example *Toxoplasma gondii* is known to synthesize SM (Azzouz *et al*., 2002) and is believed to make IPC and EPC (Welti *et al*., 2007; Sonda *et al*., 2005), but the enzymes responsible remained unknown. Similarly, IPC synthase activity is known to exist in plants but no sequence orthologues of the well studied fungal enzyme AUR1 were apparent in the genome databases (Bromley *et al*., 2003).

A functional orthologue of the fungal IPC synthase (AUR1) has been identified and characterised in the kinetoplastid, protozoan parasite *Leishmania major* (*Lmj* IPCS; Denny *et al*., 2006). This study also identified orthologues in the related parasites *Trypanosoma brucei* (*Tb* SLS 1-4) and *Trypanosoma cruzi* (*Tc* IPCS 1 and 2). However, again these enzymes remained uncharacterised despite the evidently important roles of sphingolipids in anchoring surface proteins (Sugimoto, Sakoh and Yamada, 2004), in differentiation and, perhaps, in pathogenicity (Sutterwala *et al*., 2008).

Given the fact that these enzymes represent promising drug targets, further study of the kinetoplastid and apicomplexan is essential (Denny *et al*., 2006). Such work is vital in moving towards the ultimate goal of identifying compounds with the potential for development into much needed, pharmacologically active anti-protozoals.

To move towards these goals the following objectives were set:

1. Establish the role of host sphingolipid biosynthesis in *Toxoplasma gondii* proliferation using a mutant CHO host cell line.

2. Identify and isolate the sphingolipd synthase from *T. gondii* (*Tg* SLS*)* using a bioinformatic approach.

3. Functionally characterize this enzyme (and those from the *Trypanosma* species) using an established complementation approach in yeast and various biochemical techniques.

4. Localise *Tg* SLS in the *T. gondii* parasite

5. Establish the evolutionary relationship of the apicomplexan, kinetoplastid, plant, fungal and animal sphingolipid synthases using bioinformatic and phylogenetic approaches.

CHAPTER 03

MATERIALS AND METHODOLOGY

3 MATERIALS AND METHODOLOGY

3.1 MATERIALS

Chloroform ACS reagent 99.8%, methanol ACS reagent 99.8%, isopropyl alcohol, ethanol, butanol, formaldehyde, propanol, lithium chloride ($LiCl_2$), lithium acetate (LiAc), alkaline phosphatase, 50% polyethylene glycol (PEG), potassium chloride (KCl), hydrochloric acid (HCl), acetic acid, sodium acetate, sodium dodecyl sulphate, sodium hydroxide (NaOH), sodium phosphate dibasic anhydrous, sodium chloride (NaCl), ammonium chloride (NH_4Cl), ammonium sulphate, magnesium chloride ($MgCl_2$), HEPES, pryimethamine, N, N Dimethyl acrylamide, N, N Dimethyl formamide, fuschin, Thiourea, Triton X-100, Tween 200, urea, Xanthine, EDTA, EGTA, DMSO, CHAPS, PBS, β- mercaptoethanol, 80% glycerol, agarose, agar, raffinose, glucose, sucrose, galactose, Trisma base, fatty acid free BSA, oleic acid, adenine, lycine, leucine, tryptophan, glycine, histidine amino acids, *Hs* SMS 2 primers, cyclohexamide, myriocin, amplicillin, ethidium bromide, Dulbecco's modified Eagle's medium (DMEM) and acid washed glass beads (425-600 µm, 30-40 U S sieve) were from Sigma-Aldrich®.

Protein assay kit was from BIO-RAD® with Coomassie® Brilliant Blue G-250.

Complete® EDTA-free Protease inhibitor cocktail tablets were from Roche® Applied Science.

Yeast nitrogen base, yeast extract, peptone and BODIPY® FL C_5 ceramide and NBD C_6 ceramide were from Invitrogen®.

Tryptone and yeast extract were from Melford®.

Amino acid drop-out (DO) supplement -HIS-URA-TRP was from Clontech®.

Hs SMS 1 primers, restriction enzymes and buffers were from Promega®.

Tg SLS primers, P*fu* polymerase and buffer were from Stratagene®.

Phosphatidylinositol (PI), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE) were from Avanti® Polar Lipids.

Aureobasidin A was from TaKaRa® Bio Inc.

DMEM, Ham's F12, penicillin, streptomycin, fetal bovine serum and L-glutamine were from GIBCO® BRL.

Nutridoma-SP was from Boehringer Mannheim®.

Fideli*Taq* DNA Ploymerase, T4 DNA Ligase and buffers were from GE Healthcare®.

[5,6-^{3}H]uracil (specific radioactivity 40–50 Ci/ mmol) was from New England® Nuclear Research Products.

Plasmid MiniPrep and Midi Prep, and PCR Prep kits were from QIAGEN®.

Tissue culture flasks, multiwell plates and petri dishes were from Nunc®.

Glass fibre filters were from Whatman®.

Sampling manifold for transfer of material to filters was from Millipore®.

Scintillation cocktail was BudgetSolve® from Research Products International.

Toxoplasma gondii laboratory strain RHΔHX, host VERO cells and the expression vector the pT8mycTy were a kind gift from Prof Dominique Soldati, University of Geneva.

Toxoplasma gondii genomic DNA was a kind gift from Dr Barbara Clough, MRC NIMR, London.

Trypanosoma cruzi genomic DNA was a kind gift from Dr Martin Taylor, LSHTM, London.

Trypanosoma brucei genomic DNA was a kind gift from Dr Helen Price, University of York.

Hs SMS 1 and 2 cloned cDNA was a kind gift from Dr Joost Holthuis, Utrecht University.

HFF was from the United Kingdom National Culture Collection.

SPB-1 mutants and parent CHO cells from Riken BioResource Center, Japan with the kind permission of Prof Kentaro Hanada.

YPH499 and YPH499-HIS-GAL-AURI yeast, and the pRS426MET25 yeast expression vector were kind gifts from Dr Hosam Shams-Eldin and Prof Ralph Schwarz, Phillips University, Marburg.

Arabidopsis thaliana cDNA from total flower RNA was kindly provided by Dr Tony Fawcett, Durham University.

3.2 INSTRUMENTS AND EQUIPMENT

1.5ml LoBind® tubes from Eppendorf® were used in solvent extraction steps. Eppendorf® 1.5ml centrifuge tubes were used in all other activities.

Beckman® Coulter Avanti J-20 XPI high-speed and Optima L-90K ultra-centrifuge machines were used for preparative processes. Boeco® U-320R benchtop and M-240R mirco centrifuge were used for routine centrifugation steps.

Boeco® S-32 Spectrophotometer was used in protein bioassays and culture density measurements.

MT20 Chiltern® Vortex mixture was used in mixing and cell disruption techniques.

Perkin Elmer® DNA Thermal Cycler and PCR programmer machines were used PCR amplification and programmed vector-insert ligation.

Fusion TM, Packard® Bioscience plate reader and OptiPlate-96 F from Perkin Elmer® were used in fluorescence and radiolabel quantification.

HPTLC plates were from Merck®. Visualisation and quantification of TLC were done using Fuji® FLA-3000 plate reader and interpretation was by AIDA Image Analyser software 3.52 version.

BTX® ECM630 electroporator and 2 mm gap electroporation tubes were both from Harvard Instruments.

3.3 BUFFERS, SOLUTIONS AND CULTURE MEDIA

Table 3.3.1 Buffers

Buffer	Stock solution	Volume/weight	Final concentration
STE Buffer (50 ml)	1 M Tris/ HCl pH 7.4	1.25 ml	25 mM
	1 M Sucrose	12.5 ml	250 mM
	0.5 M EDTA	100 µl	1 mM
	Complete® protease inhibitor	1 tablet	
		Add 50 ml water	
Storage Buffer (50 ml)	1 M Tris/ HCl pH 7.4	2.5 ml	50 mM
	80% (w/v) Glycerol	6.25 ml	10% (w/v)
	1 M $MgCl_2$	0.25 ml	5 mM
	Complete® protease inhibitor	1 tablet	
		Add 50 ml water	
Tris/EDTA/BSA or Assay Buffer (50 ml)	1 M Tris/ HCl pH 7.4	12.5 ml	250 mM
	0.5 M EDTA	2.5 ml	25 mM
	Fatty acid free BSA	750 mg	15 mg/ ml
		Add 50 ml water	
PBS (100 ml)	PBS from Sigma-Aldrich®	1 tablet	
		Add 100 ml water	

Table 3.3.1 Buffers (Contd)

Buffer	Stock solution	Volume/weight	Final concentration
10X TE Buffer (1 L)	1 M Tris/ HCl (pH 7.4)	100 ml	
	0.5 M EDTA (pH 8.0)	20 ml	
		Add 880 ml ultra-pure water	
Tris/ $MgCl_2$ / Triton X-100 buffer	1 M Tris/ HCl (pH 7.4)	1 ml	20 mM
(Sphingomyelinase assay buffer)	1 M $MgCl_2$	0.5 ml	10 mM
	Triton X-100	0.025 g	0.05% (w/v)
		Add water up to 50 ml	

Table 3.3.2 Solutions and reagents

Solution/ reagent	Stock solution	Volume/weight
10X TE/ LiAc (50 ml)	1 M Lithium Acetate	5 ml
	10 X TE Buffer	5 ml
		Add 50 ml ultra-pure water
10X PEG/ LiAc (50 ml)	1 M Lithium Acetate	5 ml
	10X TE Buffer	5 ml
	50% PEG 3350	50 ml
Cytomix	120 mM KCl 0.15 mM $CaCl_2$ 10 mM K_2HPO_4 (pH 7.6) 25 mM HEPES (pH 7.6) 2 mM EGTA 5 mM $MgCl_2$	Filter sterilized

Table 3.3.3 Culture media

Media		Stock	Volume/weight
SD-HIS-URA (1 L)	Solution I	Glucose	20 g
			Add 820 ml of water and autoclaved
		Add 20 g of Agar for SDA-HIS-URA solid medium	
	Solution II A	Yeast Nitrogen base	1.7 g
		NH_4SO_4	5 g
			Add 80 ml of water and autoclaved
	Solution II B	Amino acid Drop out supplement (no Histidine, Uracil or Tryptophane)	0.7 g
		Tryptophane	4 mg
	Solution II A and II B were mixed and added to solution I (When temperature falls below 45°C, immediately poured over the plates under aseptic conditions for solid medium)		
SD-HIS-URA-MET (1 L)	Solution I	Glucose	20 g
			Add 820 ml of water and autoclaved
		Add 20 g of Agar for SDA-HIS-URA solid medium	
	Solution II A	Yeast Nitrogen base	1.7 g
		NH_4SO_4	5 g
			Add 90 ml of water and autoclaved
	Solution II B	2 mg/ ml Adenine	2 ml
		10 mg/ ml Lysine	3 ml
		10 mg/ ml Leucine	3 ml
		10 mg/ ml Tryptophane	2 ml
			Filter sterilized
	Solution II A and II B were mixed and added to solution I (When temperature falls below 45°C, immediately poured over the plates under aseptic conditions for solid medium)		

Table 3.3.3 Culture media (contd)

Media		Stock	Volume/weight
SGR-HIS-URA (1 L)	Solution I	Galactose	40 g
		Raffinose	20g
			Add 720 ml of water and autoclaved
		Add 20 g of Agar for SGRA-HIS-URA solid medium	
	Solution II A	Yeast Nitrogen base	1.7 g
		NH_4SO_4	5 g
			Add 80 ml of water and autoclaved
	Solution II B	Amino acid Drop Out (DO) supplement (no Histidine, Uracil or Tryptophane)	0.7 g
		Tryptophane	4 mg
			Add 100 ml of water and filter sterilized
	Solution II A and II B were mixed and added to solution I (When temperature falls below 45°C, immediately poured over the plates under aseptic conditions for solid medium)		
YPGR (1L)	Solution I	Yeast extract	10 g
		Peptone	20 g
			Add 800 ml of water and autoclaved
	Solution II	Galactose	40 g
		Raffinose	20g
			Add 200 ml of water and autoclaved
	Solution I and II were mixed under aseptic conditions		

Table 3.3.3 Culture media (contd)

Media		Stock	Volume/weight
YPD (1 L)	Solution I	Yeast extract	10 g
		Peptone	20 g
			Add 950 ml of water and autoclaved
		Add 20 g of Agar for YPDA solid medium	
	Solution II	40% stock solution of Dextrose	50 ml (2%)
	When Solution I cooled down to 55°C, Solution II was added under aseptic conditions.		
	(The medium was poured over the plates immediately under aseptic conditions.)		
PLB (1L)		Tryptone	10 g
		Yeast extract	5 g
		NaCl	10 g
			Add 1 L of water and autoclaved. pH 7.5
		Add 20 g of Agar for PLB agar medium	

Table 3.3.3 Culture media (contd)

Media	Stock	Volume/weight
SOC (1L)	Tryptone	20 g
	Yeast extract	5 g
	NaCl	0.584 g
	KCl	0.186 g
	$MgCl_2$	2.003 g
	$MgSO_4$	2.265 g
	Glucose	3.6 g
		Add 1 L of water and autoclaved

Table 3.3.3 Culture media (contd)

Media		Stock	Volume/weight
GYT (100 ml)		10% Glycerol	
		0.125% TE	
		0.25% Tryptone	Add up to 100 ml of water
SD 2% Glucose/ 0.8% Agarose (8:1:1) (1 L)	Solution I	Glucose	20 g dissolved in 800 ml of water and autoclaved
		Agarose	8 g dissolved in 100 ml of water and autoclaved
	Solution II A	Yeast Nitrogen base	1.7 g
		NH_4SO_4	5 g
			Add 90 ml of water and autoclaved
	Solution II B	2 mg/ ml Adenine	2 ml
		10 mg/ ml Leucine	3 ml
		10 mg/ ml Lysine	3 ml
		10 mg/ ml Tryptophane	2 ml
			Filter sterilized
	Solution II was added 1:10 to Solution I		

Table 3.3.3 Culture media (contd)

Media	Stock	Volume/weight
DMEM (1L)	DMEM stock solution	500 ml
	FBS	50 ml
	Streptomycin/penicillin	5 ml
	2 mM glutamine	
		Mixed in laminar floor hood and filter sterilized

3.4 BIOINFORMATIC STUDY

3.4.1 Identification of hypothetical enzymes Tg SLS and At IPCS

Two candidate sphingolipid synthase coding sequences were previously identified in the complete genome database of the malaria parasite *Plasmodium falciparum* (plasmodb.org; Huitema *et al*., 2004). A WU-BLAST search (Gish, 1996-2001) http://blast.wustl.edu) was performed on the then incomplete *Toxoplasma gondii* genome database (toxodb.org).

A single sequence orthologue was identified, *Tg* SLS accession number 50.m03164. The identified *Leishmania major* enzyme (*Lmj* IPCS; Denny *et al*., 2006) was used in BLAST search of the *Arabidopsis thaliana* genome sequence database (The Institute for Genomic Research) with the previously identified *L. major* IPCS (Denny *et al*., 2006). Three closely related candidates were identified: *Arabidopsis thaliana* IPCS 1-3, accession numbers At3g54020.1, At2g37940.1 and At2g29525.1.

3.4.2 Membrane topology prediction of hypothetical protein of Tg SLS

PHD protein prediction program (Expasy.ch) was used to predict the membrane topology of the identified hypothetical protein, *Tg* SLS.

3.4.3 Sequence alignment and phylogenetic analysis

Protein sequence alignments were performed using Clustal W (Jeanmougin *et al*., 1998). Phylogenetic analyses were performed on edited alignments using maximum parsimony (PHYLIP Phylogeny Inference Package, version 3.5) constructed with maximum parsimony using Phylip according to author's instructions.

3.5 AMPLIFICATION AND CLONING OF SPHINGOLIPID SYNTHASE OPEN READING FRAMES

3.5.1 Tg SLS

The 50.m03113 open reading frame was amplified from genomic *T. gondii* using *Pfu* polymerase (Appendix I, 1.1.1) and the 1 μl of each of the primer pairs:

5' *Tg* SLS EcoRI	cgcgaattcATGCCCAGAACAGAGATG
3' *Tg* SLS HindIII	cccaagcttTTAGAGTCCCTCGATGGCGCGAACGAT
or	
5' *Tg* SLS EcoRI	cgcgaattcATGCCCAGAACAGAGATG
3' *Tg* SLS NsiI	cccatgcatTTAGAGTCCCTCGATGGCGCGAACGATG

Cloning sites are shown in lower case, with coding sequence in upper case.

The product was identified by running a 0.8% agarose gel impregnated with ethidium bromide. Positive and negative controls were run alongside using *Tg* SPT and water respectively.

The amplified DNA product was purified with QIAGEN® PCR Prep kit and digested (with restriction enzymes Bam HI and Hind III or EcoRI and NsiI according to manufacturers' instructions (Appendix I, 1.2)). Digested DNA was then purified again using the PCR Prep kit. In parallel, the yeast expression vector pRS426MET25 (Appendix II) was digested with Bam HI and Hind III whilst the *T. gondii* expression vector pT8mycTyGFP (Appendix III) was digested with EcoRI and NsiI, both were fractionated on an agarose gel and purified using the PCR Prep Kit. The amplified *Tg* SLS were ligated into these using

T4 ligase according to the manufacturer's conditions (Appendix I, 1.3), creating pRS426*Tg* SLS and pT8myc*Tg* SLSTy. These were transformed into XL-1 blue super competent cells (Stratagene®) according to manufacturers' instruction and selected on PLB plates with 25 µg/ µl of amplicillin incubated at 37 °C overnight. A negative control was performed with 2 µl water instead of DNA. Individual colonies were picked and grown in 3 ml of PLB liquid medium with 25 µg/ µl of amplicillin at 37 °C overnight. Plasmid purification using the QIAGEN® Miniprep was done according to the manufacturer's protocol and DNA screen for the presence of the inserts by restriction digest.

3.5.2 At IPCS

At IPCS 1 was amplified from *Arabidopsis* cDNA (from total flower RNA) using Fideli*Taq* (Appendix I, 1.1.3) and the primer pair:

5' *At* IPCS 1 BamHI cgcggatccATGACGCTTTATATTCGCCGCG

3' *At* IPCS 1 EcoRI ccggaattcTCATGTGCCATTAGTAGCATTATC

As above the product was ligated into the yeast vector pRS426MET25 (Appendix I, 1.3 and Appendix II) using the restriction sites shown, creating pRS426*At* IPCS 1, and clones selected. Due to the infidelity of cDNA production the plasmids selected were sequenced (MWG) to verify the coding region. Cloning sites are shown in lower case, with coding sequence in upper case. *At* IPCS 2 and 3 were similarly cloned in the vector by Yoko Okada (Durham University).

3.5.3 Tc IPCS

Tc IPCS was amplified from *Trypanosoma cruzi* genomic DNA using Fideli*Taq* (Appendix I, 1.1.3) and the primer pair:

5' *Tc* IPCS BamHI cgcggatccATGGTTTTAATGGGGCCTCATTCTGC

3' *Tc* IPCS HindIII cccaagcttTTATCACCAGTGTCCAAAATTCCCAC

As above the product was ligated into the yeast vector pRS426MET25 (Appendix II) using the restriction sites shown, creating pRS426*Tc* IPCS, and clones selected. Given the near identity of the 2 isoforms of the enzyme (Sevova *et al*., 2010) the insert could have represented either *Tc* IPCS 1.1 or *Tc* IPCS 1.2. Sequence analyses (MWG) confirmed the chosen cloned to contain *Tc* IPCS 1.2, the vector being pRS426*Tc* IPCS 1.2. Cloning sites are shown in lower case, with coding sequence in upper case.

3.5.4 Tb SLS 1 and 4

Tb SLS 4 was amplified from *Trypanosoma brucei* genomic DNA using P*fu* polymerase (Appendix I, 1.1.1) and the primer pair:

5' *Tb* SLS1000 EcoRI ccggaattcATGATTAGTTACCCTTTCTTCTCCC

3' *Tb* SLS1000 XhoI ccgctcgagTCACACATACGCCCCACATTTAAAC

As above the product was ligated into the yeast vector pRS426MET25 using the restriction sites shown (Appendix I, 1.3 and Appendix II), creating pRS426*Tc* IPCS, and clones selected. Cloning sites are shown in lower case, with coding sequence in upper case. *Tb* SLS 1 was similarly cloned by Pan Ssu-Ying (Durham University).

3.5.5 Hs SMS 1 and 2

Hs SMS 1 and 2 were amplified from sequenced cDNA clones using P*fu* polymerase (Appendix I, 1.1.1) and the primer pairs:

5' *Hs* SMS1 BamHI	cgcggatccATGAAGGAAGTGGTTTATTG
3' *Hs* SMS1 EcoRI	ccggaattcTTATGTGTCATTCACCAGCCGG
or	
5' *Hs* SMS2 BamHI	cgcggatccATGGATATCATAGAGACAGCAAAAC
3' *Hs* SMS2 EcoRI	ccggaattcTTAGGTCGATTTCTCATTGTCTTC

As above the products were ligated into the yeast vector pRS426MET25 using the restriction sites shown (Appendix I, 1.3 and Appendix II), creating pRS426*Hs* SMS 1 and 2, and clones selected. Cloning sites are shown in lower case, with coding sequence in upper case.

3.6 TRANSFORMATION OF PRS426 BASED VECTORS INTO YEAST

In the YPH499-HIS-GAL-AUR1 auxotrophic *S. cerivisiae* strain, expression of the essential AUR1 is suppressed in the presence of glucose (Denny *et al*., 2006). Therefore, mutant cells were grown in YPGR (Table 3.3.3) (wild type YPH499 in YPD (Table 3.3.3)) before harvesting during their exponential growth phase by centrifugation. Then the cells were re-suspended in sterile water, vortexed for 1 minute and washed by centrifuging. The pellet was re-suspended in 250 µl of freshly prepared 1XTE / LiAc (Table 3.3.2) and transferred into an Eppendorf® tube. 100 µl of this cell suspension was mixed with 1 µl of a pRS426 based vector and 10 µl of Herring testis carrier DNA. 600 µl of freshly prepared sterile PEG / LiAc (Table 3.3.2) was added to each tube, vortexed and incubated at 30 °C

for 30 minutes. 70 µl of sterile DMSO were added and mixed by gentle inversion of the tubes. The tubes were heat shocked by placing in a hot water bath of 42 °C for 15 minutes and chilled on ice for 1-2 minutes immediately. The cells were pelleted by a quick spin and re-suspended in 500 µl of 1X TE. 100 µl of the cell suspension were spread over SGRA-HIS-URA or SDA-HIS-URA plates (Table 3.3.3) and incubated at 30°C for 4-5 days until the colonies appear. A negative control was performed with water instead of plasmid DNA. Individual colonies were then inoculated in SD-HIS-URA liquid medium (Table 3.3.3). A smear of cell culture was streaked out on SDA-HIS-URA (Table 3.3.3) plates for further use. Glycerol stocks were prepared by adding glycerol to 15% in a liquid culture, mixing and freezing at -80°C (Appendix I, 1.4).

3.7 METABOLIC LABELLING YEAST WITH NBD C_6 CERAMIDE OR FL C_5 CERAMIDE

The sphingolipid synthase complemented YPH499-HIS-GAL-AUR1 yeast was cultured in SD-HIS-URA medium at 30 °C overnight. YPH499-HIS-GAL-AUR1 expressing *Hs* SMS 1 or 2 (not complemented) were cultured in SGR-HIS-URA medium at 30 °C overnight. 2.5 OD_{600} units harvested by centrifugation and resuspended in either SD or SGR (Table 3.3.3) with 5 µM FL C_5 ceramide or NBD C_6 ceramide conjugated with 5 µM defatted BSA prepared according to the manufacturer's protocol (Appendix I, 1.5). After incubation at 30 °C for 1 hour, the yeast was washed twice with SD-HIS-URA or SGR-HIS-URA (Table 3.3.3). Cells were resuspended in C:M 1:1 (v/v) and homogenised using acid washed glass beads. The membrane lipids were then extracted into the organic solvents by bi-phasic separation (Fischl *et al.*, 2000) and cell membrane debris removed by centrifugation. The contents in the organic phase were concentrated in the Rotavapor Eppendorf® concentrator 5301 at 30 °C for 20 minutes. The dried pellet was re-suspended

in 20 µl of C:M:W 10:10:03 (v/v/v). Then a TLC assay was carried out and analysed as described below (3.11) and previously (Denny *et al*., 2006).

3.8 SPHINGOMYELINASE ASSAY

Lipid extracts of NBD C_6 ceramide metabolically labelled yeast was dried with 50 µg of sphingomyelin in the Eppendorf® concentrator. The dried lipid was then reuspended in 600 µl of 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and 0.05% (w/v) Triton X-100 (Table 3.3.1). The suspensions were divided equally in to two LoBind® tubes and 0.25 µl of *B. cereus* sphingomyelinase added. Tubes were incubated at 37 °C for 2 hours with continuous shaking. 1 µl of acetic acid was added to stop the reactions. 600 µl of C:M:W (10:10:03 v/v/v) were added and the organic layer was extracted and analysed as above (3.7).

3.9 METABOLIC LABELLING OF *TOXOPLASMA GONDII*

Parasites were maintained in VERO cells (kidney epithelial cells from the African Green Monkey) grown in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2 (Weiss and Kim, 2008). To purify parasites infected host cells were disrupted by passing through a narrow gauge needle and the milieux filtered through 3 and 5 µm polycarbonate filters (Millipore®) to remove contaminating host material. *T. gondii* were isolated by centrifugation at 2877 x g for 5 minutes at room temperature, re-suspended in DMEM (Table 3.3.3) without serum, washed and counted using a haemocytometer. 10^7 parasites were resuspended in 1 ml of serum-free DMEM before incubation for 30 minutes at 37 °C, subsequently BSA conjugated FL C_5 ceramide or NBD C_6 ceramide (Appendix I, 1.5) was added at 5 µM and the sample incubated at 37 °C for a further 2 hours**.** Parasites

were then pelleted by centrifugation and washed twice with DMEM. The products were extracted using the bi-phasic separation method and the lipid fraction analysed as above (3.7).

3.10 METABOLIC LABELLING OF VERO MAMMALIAN CELLS

The VERO cells were scraped from tissue culture flasks, re-suspended in serum-free DMEM without BSA and washed with centrifugation at 957 x g for 3 minutes at room temperature. $3x10^5$ cells were then labelled and lipids prepared as above (3.9).

3.11 THIN LAYER CHROMATOGRAPHY (TLC)

The fluorescence of the labelled lipid samples was measured at 480 nm (Fusion® TM, Packard Bioscience) against a 100 µl of methanol as a blank. Equilibrated samples were then loaded onto high performance (HP) TLC silica plates. Fractionation was performed in a solvent phase of C:M:0.25% KCl, 55:45:10 (v/v/v) for 2 hours 30 minutes. The plate was scanned using the Fuji® FLA 3000 and analysed by AIDA Image, FLA software programs.

3.12 MICROSOMAL FRACTIONATION OF COMPLEMENTED YEAST

The *Tg* SLS complemented HIS-GAL-AUR1 were grown in volumes of at least 500 ml in SD-HIS-URA medium (Table 3.3.3) at 30 °C over a period of 3-4 days until OD_{600}= 0.6-0.7. The cells were then harvested by centrifuged at 4785 x g for 15 minutes at 4 °C and the pellet washed with ice-cold PBS (Table 3.3.1). They were then re-suspended in 50 ml of cold PBS and pelleted by centrifugation and the pellet weighed. 1 ml of STE buffer with protease inhibitor (Table 3.3.1) was added to the pellet with approximately 0.5 ml of acid washed glass beads. The cells were homogenised by vortexing for 1 minute and placed on

ice for a further minute, this was repeated 10 times over a period of 20 minutes. The homogenate was centrifuged at 4785 x g for 10 minutes and the supernatant removed to a 4 ml poly-allomer centrifuge tube and kept in 4 °C. 1 ml of STE buffer (Table 3.3.1) was added to the pellet again and homogenised as previously. This procedure was repeated until the poly-allomer tube was full. The preparation was subsequently centrifuged at 27000 x g for 35 minutes at 4 °C in the Beckman® L8-70M ultracentrifuge. The supernatant was removed to a new poly-allomer centrifuge tube and ultra-centrifuged at 105000 x g for 1 hour 45 minutes at 4 °C. The supernatant was removed and the pellet air dried then resuspended in 50 µl of cold storage buffer (Table 3.3.1) before storage at -80 °C until further use (Figueiredo *et al*., 2005).

3.13 ESTIMATION OF PROTEIN CONTENT OF MICROSOMAL FRACTION

1 µl of microsomal preparation was diluted in 800 µl of water. 200 µl of BioRad® bioassay dye reagent was added to each and mixed by inverting the tubes for a few turns. The absorbance was measured using Boeco® S-30 spectrophotometer at 595 nm against a blank. The protein concentration was determined by reference to a BSA standard curve and adjusted to 10 mg/ ml.

3.14 CHAPS WASHING OF MICROSOMES

Microsomes at 10 mg/ ml were extracted in 2.5% (40 mM) of CHAPS for 1 hour on ice (Mina *et al*., 2010 b) in 4 ml poly-allomer centrifuge tubes. The tubes were topped up with STE buffer with protease inhibitor (Table 3.3.1) and ultra-centrifuged at 105000 x g for 1 hour 45 minutes at 4 °C. The pellet was re-suspended in 50 µl of cold storage buffer (Table

3.3.1). The protein concentration was determined as above (3.13) and adjusted to 10 mg/ ml before storage at -80 °C until further use.

3.15 *IN VITRO TG* SLS MICROSOMAL ASSAY

5 µl of 10 mM PI, 4 µl of 13 mM PE and 4 µl of 13 mM PC were air dried for 10-15 minutes in LoBind® (lipid low binding) centrifuge tubes. 20 µl of Assay Buffer (Tris/EDTA/BSA) (Table 3.3.1) were added to each including a blank as a control. The tubes were sonicated for 2 minutes and kept on ice. 2 µl of crude or CHAPS washed microsomal extract (20 µg protein) were added to each and the final volume was adjusted to 48 µl with water. 2 µl of 5 µM FL C_5 ceramide or 2 µl of 100 µM NBD C_6 ceramide were added to each tube and incubated at 30 °C for 1 hour. 150 µl of C:M:W 10:10:03 (v/v/v) were added and the organic layer extracted by bi-phasic separation before TLC analyses as described above.

3.16 AUREOBASIDIN A SENSITIVITY ASSAY

4 µl of 10 mM PI were added to 1.5 ml LoBind® tubes and air-dried for 15 minutes. 20 µl of Tris/EDTA/BSA buffer (Table 3.3.1) was added and the tubes sonicated for 2 minutes before chilling on ice. Appropriate concentrations (0 µM, 10 µM, 25 µM, 50 µM, 75 µM, 100 µM, 125 µM, and 150 µM) of aureobasidin A in DMSO were added to the tubes. 2 µl (20 µg protein) of CHAPS-washed microsomes were added to each and the final volume of the reaction mixture was adjusted to 48 µl with sterile water. This was incubated at 30 °C for 30 minutes before the addition of 2 µl of 100 µM NBD C_6 ceramide and further incubation at 30 °C for 1 hour. 150 µl of C:M:W 10:10:03 (v/v/v) were added and the organic layer extracted by bi-phasic separation before TLC analyses as described above.

3.17 AGAR DIFFUSION ASSAY

Complemented yeast cells were grown in SD-HIS-URA and harvested in the exponential phase of growth. 2.4 x 10^7 (OD_{600}=1 unit=10^7) cells were resuspended in 14 ml of warm (45 °C) SD-HIS-URA solution I with 2% agarose and 1 ml of Solution II added (Table 3.3.3). After gentle mixing the solution was immediately poured over petri dishes and allowed to set under aseptic conditions. Then the appropriate volumes (1 µl, 2 µl and 3 µl) and concentrations (prepared in DMSO) of aureobasidin A (25 µM, 100 µM), cyclohexamide (50 µM), myriocin (1 mM) were dropped on to the plates with DMSO as a control before incubation for 3-5 days at 30 °C.

3.18 INVASION AND PROLIFERATION ASSAYS

3.18.1 Cell culture and infection

CHO-K1, temperature sensitive SPT mutant SPB-1 and SPB-1/cLCB-1 cells were grown in Ham's F-12 media supplemented with 10% FBS at 37°C or 33°C (permissive temperature) plus 5% CO_2. For induction of the temperature-sensitive phenotype 10^5 cells were seeded into 24-well plates and incubated at 33°C for 24 hours. The medium was then replaced with Ham's F12 medium supplemented with 10% FBS or, to form serum reduced media, 0.1% FBS, 1% Nutridoma-SP and 250 µM oleic acid, 5% fatty acid free BSA. The low-serum medium contains less than 1 µM sphingomyelin (Hanada *et al*., 1992). Myriocin was added in this phase as indicated and cell viability was quantified by Trypan Blue exclusion. Cells were then incubated at 39 °C for 72 hours before *T. gondii*, purified as above (3.9), were added at a ratio of 2 parasites to 1 host cell and allowed to invade for 4 hours. Subsequently, after washing with PBS, cells were incubated for a further 24 hours in normal or serum reduced media before assay.

3.18.2 Invasion assay

To establish the ability of the parasites to invade the various host cells under the experimental conditions the cells were fixed with methanol at -20°C for 10 minutes, dried, stained with 300 nM DAPI in PBS for 5 minutes and rinsed with PBS. Visualization on an Olympus® 1X-71 epifluorescent microscope allowed invasion to be scored in 100 host cells.

3.18.3 Proliferation assay

After the 4 hour invasion time 1.0 μ Ci of [5,6-^{3}H] uracil was added per well and cultures incubated for 24 hours as before. Incorporation of [^{3}H] uracil into trichloroacetic acid (TCA)-precipitable material was measured after this period. Supernatant was removed from the cells by aspiration, and then they were solubilised with 800 μl of 1% (w/v) SDS containing 100 μg of unlabelled uracil/ ml. 2.4 ml of 0.3 M TCA was then added and, after a 15 minute incubation at 4 °C, precipitates were collected on to glass fibre using a sampling manifold. The filters were washed twice with 0.3 M TCA and once with 95% ethanol, dried, placed in 10 ml of scintillation cocktail and the incorporated radioactivity then measured. Confluent non-infected cells incorporated very little [^{3}H] uracil and the values of these were subtracted from the readings with infected cells to ensure measurements reflected parasite proliferation.

3.19 IMMUNOLOCALISATION OF *TG*SLS

3.19.1 Transfection of Toxoplasma gondii

Parasites were maintained in VERO cells and isolated by filtration as described above (3.9). After counting using a haemocytometer the *T. gondii* cells were transiently transfected with pT8myc-*Tg* SLS-Ty (Appendix III) as described by Weiss and Kim (2008). Briefly, parasites were resuspended in complete cytomix at 3.3 x 10^7/ ml. 300 μl of this was then mixed with 100 μl of plasmid DNA (50-100 μg) in cytomix in a 2 mm gap electroporation cuvette. This was electroporated with a single pulse of 1.5 kV, a resistance setting of 25 Ω and a capacitor setting of 25 μF using a BTX BCM630 electroporator. Parasites were transferred to the confluent VERO cell cultures in 12 well plates with coverslips and incubated for 24-36 hours at 37 °C.

3.19.2 Immunolocalisation

Transfected intracellular parasites were fixed with a solution of 1 ml of formaldehyde (37%), 0.4 g sucrose and 19 ml of water for 30 minutes at room temperature. Cells were blocked and permeabilised in 2.5% FBS and 0.2% Triton X 100 in PBS for 30 minutes. The primary antibody (anti-Ty antibody, BB_2) was prepared at a 1:100 concentration in 2.5% FBS and 0.1% Triton X100 and applied to the cells for 1 hour at room temperature. Coverslips were then washed 3 times with PBS for 5 minutes each and the secondary antibody, anti-mouse IgG (594 Alexa Fluor), applied (1:100 in 2.5% FCS and 0.1% Triton X100) for 1 hour. After washing as before the coverslips were stained with 300 nM DAPI in PBS for 5 minutes and rinsed with PBS. Coverslips mounted on a slide with 5 μl of mounting solution and the edges sealed with a nail varnish before visualisation on an Olympus® 1X-71 epifluorescent microscope and image capture using QImaging Retiga

camera and OpenLab Improvison software. Images were subsequently manipulated using Adobe Photoshop.

CHAPTER 04

THE ROLE OF HOST CELL SPHINGOLIPID BIOSYNTHESIS IN *T. GONDII* PROLIFERATION

4 THE ROLE OF HOST CELL SPHINGOLIPID BIOSYNTHESIS IN *T. GONDII PROLIFERATION*

Although the parasitophorous vacuole (PV) does not intersect directly with the host vesicular trafficking pathways, *Toxoplasma gondii* remains dependent on host cells for a number of critical nutrients. Significant progress has been made in the understanding of the mechanisms *T. gondii* uses to scavenge nutrients from its host, especially in the case of lipid molecules (Bottova *et al*., 2009). For example, it appears likely that when synthesis of host phosphatidylcholine (PC, a phosphoglycerolipid) is restricted *T. gondii* scavenge host choline and synthesize PC *de novo* (Charron and Sibley, 2002).

T. gondii has also been demonstrated to synthesize sphingolipids *de novo* - the incorporation of tritiated serine into sphingolipid species demonstrated the presence of an active serine palmitoyltransferase (*Tg* SPT) (Azzouz *et al*., 2002; Pratt *et al*., in preparation). Serine was also incorporated into sphingomyelin (SM) and glycosphingolipid (GSL) species (Azzouz *et al*., 2002). However, the role of sphingolipids in host-pathogen interactions and pathogenesis remains unknown.

To begin to understand the role of host sphingolipid synthesis in *T. gondii* pathogenesis Chinese Hamster Ovary (CHO) cells with a temperature sensitive (ts) SPT were used as hosts (Hanada *et al*., 1990). In this cell line, SPB-1, the LCB1 subunit of SPT is thermolabile and sphingolipids are depleted approximately 99% at the non-permissive temperature (39 °C) when the cells are grown with reduced sera. Similar studies have demonstrated that the bacterium *Chlamydia trachomatis* is unable to replicate within these cells showing that host biosynthesis of sphingolipids is essential for intracellular growth of this pathogen (van Ooij *et al*., 2000).

Proliferation of *T. gondii* within SPB-1 cells, and the parental and LCB1 cDNA complemented lines as controls, was measured as described in Materials and Methodology. Briefly, before invasion, host cells were incubated for 24 hours in 10% FCS HAM media followed by 72 hours in 10% FCS or serum-reduced HAM media. In the same media *T. gondii* (strain RH, 2 parasites to 1 host cell) were then allowed to invade for 4 hours before washing and incubating for a further 24 hours. *T. gondii* proliferation was established by measuring [^{3}H]-uracil incorporation (into RNA) during this time period, host incorporation into non-infected cells was subtracted. Notably, because they are confluent and non-dividing incorporation of uracil into host cells is very low. To establish the role of host sphingolipids in *T. gondii* cell invasion, the numbers of infected cells were scored using microscopy after staining DNA using DAPI. Such experiments determined whether host sphingolipid biosynthesis is important for invasion and proliferation of *T. gondii*.

All experiments were performed in triplicate at the SPB-1 permissive (33 °C) and non-permissive (39 °C) temperatures. All results normalised with respect to controls-proliferation and invasion in parental CHO cells in the presence of 10% FCS.

Figure 4-1: Cells were cultured in 10% FCS or in serum reduced media at the permissive temperature (33 °C). *T. gondii* proliferation in the SPT-compromised host cells (SPB-1) and control lines (CHO-K1 and SPB-cLCB1) was established. No difference in parasite proliferation was apparent in the three host cell lines. *T. gondii* proliferation was monitored by incorporation of [^{3}H] uracil. All results were normalised with respect to *T. gondii* proliferation in wild type CHO-K1 cells in the presence of 10% FCS and expressed as % of CHO-K1 in 10% FCS media on the *y* axis.

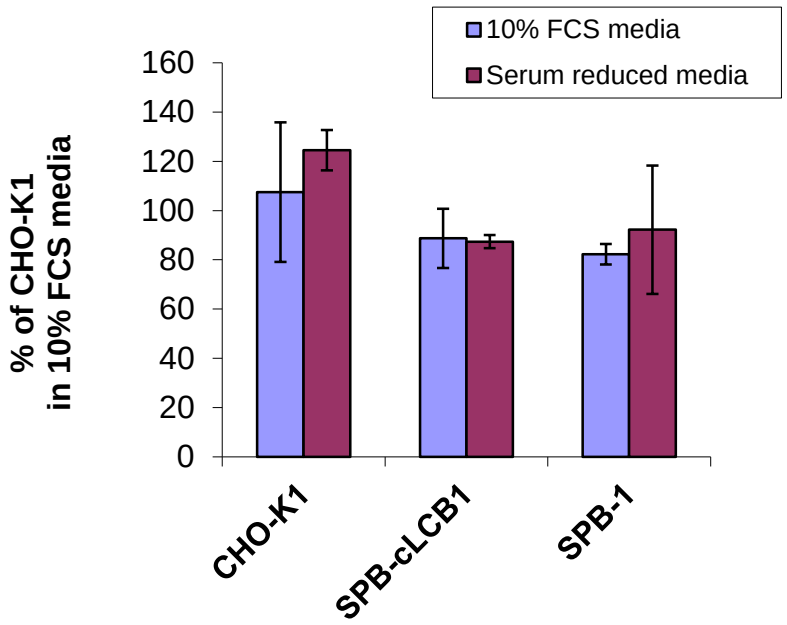


Figure 4-2: Cells were cultured in 10% FCS or in serum reduced media at the non-permissive temperature (39 °C). In the serum reduced media parasite proliferation in the SPT- compromised host cells (SPB-1) was decreased compared to the control lines (CHO-K1 and SPB-cLCB1). This effect was ablated by the presence of 10% FCS. All results normalised with respect to proliferation in parental CHO cells in the presence of 10% FCS. *T. gondii* proliferation was monitored by incorporation of [^{3}H] uracil. All results were normalised with respect to *T. gondii* proliferation in wild type CHO-K1 cells in the presence of 10% FCS and expressed as % of CHO-K1 in 10% FCS media on the *y* axis.

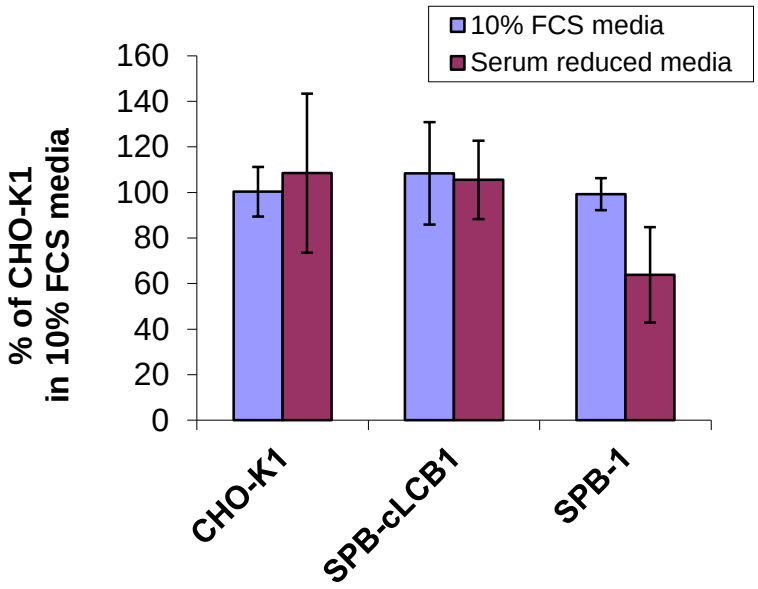


Figure 4-3: Repeating the above experiment in the serum reduced media at the non-permissive temperature of 39 °C but in the presence or absence of 50 μM myriocin, a specific inhibitor of the mammalian SPT. Myriocin at this concentration was shown to be non-toxic under the assay conditions by Trypan Blue exclusion assay (data not shown). *T. gondii* proliferation was monitored by incorporation of [^{3}H] uracil. All results were normalised with respect to *T. gondii* proliferation in wild type CHO-K1 cells in the presence of 10% FCS and expressed as % of CHO-K1 in 10% FCS media on the *y* axis. Myriocin inhibits host SPT, similar effect to SPB-1 mutant. Notably, in the presence of this inhibitor reduced parasite proliferation in the parental and complemented CHO cells to levels seen in the SPB-1 mutant. Myriocin had no effect on proliferation in the mutant CHO cells.

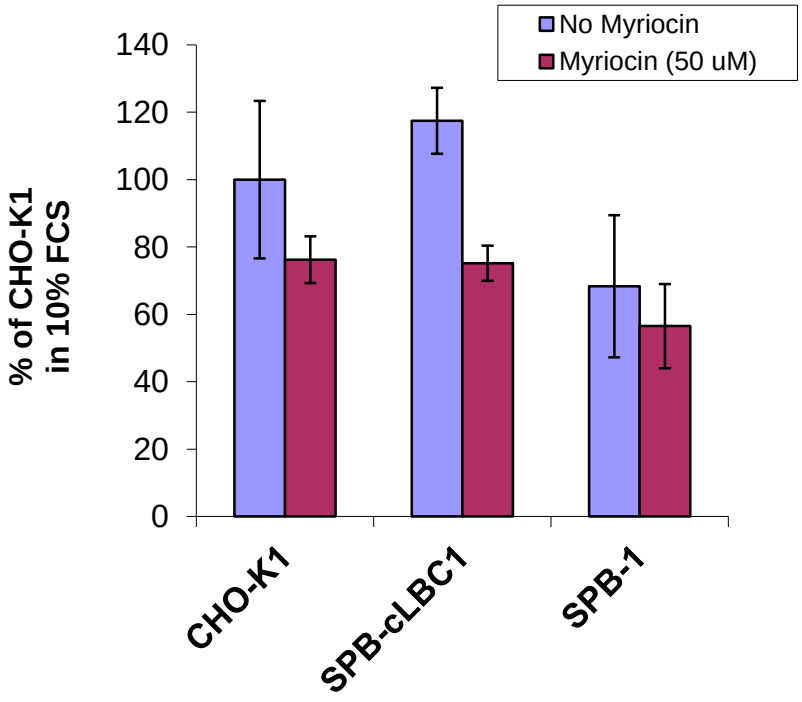
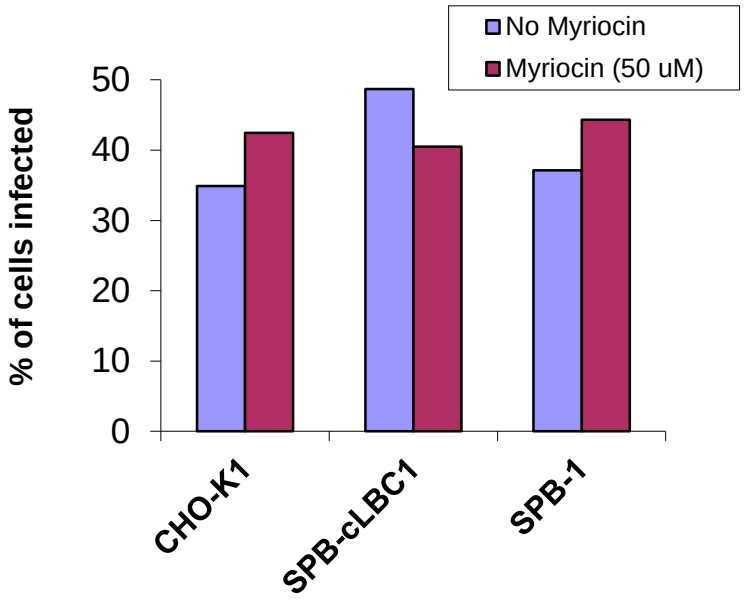


Figure 4-4: To ensure that the effects observed above were due to the role of host sphingolipid biosynthesis on proliferation, rather than a defect in invasion, the numbers of infected cells was established under the same conditions as above in serum reduced media at the non-permissive temperature of 39 °C. The effects of 50 μM myriocin were also established. After DAPI staining and microscopy as described above, 100 host cells of each line were scored for the presence or absence of *T. gondii* parasites. The results are expressed as % of cell infected on the *y* axis. This clearly demonstrated that host SPT function and sphingolipid biosynthesis play no apparent role in invasion and the differences observed above were due to a defect in proliferation.



SUMMARY

Taken together these results indicate that host sphingolipid biosynthesis plays a role in parasite proliferation but not invasion. Although the presence of large quantities of sera, and the complex lipids contained within, ablates this indicating that the *T. gondii* is able to scavenge sphingolipid from the extracellular environment. However, even in reduced sera media proliferation was still ~70% of that in wild type and complemented mutant CHO cells indicating that the parasite *de novo* sphingolipid biosynthetic pathway is likely to play an important role in pathogenicity.

CHAPTER 05

EXPLORING *T. GONDII* COMPLEX SPHINGOLIPID BIOSYNTHESIS

5 EXPLORING *T. GONDII* COMPLEX SPHINGOLIPID BIOSYNTHESIS

As discussed above *T. gondii* has been demonstrated to synthesize sphingolipids *de novo* with the tritiated serine being incorporated into complex sphingolipid proposed to be incorporated into the phosphosphingolipid SM (Azzouz *et al*., 2002). The presence of SM was later confirmed in a lipidomic analysis using mass spectrometry although it is less prominent than in host mammalian cells (Welti *et al*., 2007). This approach also uncovered ethanolamine phosphorylceramide (EPC) as a relatively abundant phosphosphingolipid in the parasites, approximately 100-fold more abundant than in the host where this lipid species is rare (Welti *et al*., 2007). However, it should be noted that this work could not distinguish between parasite lipids synthesized *de novo* and those scavenged from the host cell (Welti *et al*., 2007). In a further study, employing metabolic labelling, the non-mammalian phosphosphingolipid, inositol phosphorylceramide (IPC), was proposed to be present (Sonda *et al*., 2005). Despite this progress the identity of the enzyme(s) responsible for the synthesis of the *T. gondii* phosphosphingolipid remained unknown.

5.1 IDENTIFICATION OF THE *T. GONDII* SPHINGOLIPID SYNTHASE (*TG* SLS)

5.1.1 Identification of the hypothetical protein Tg SLS

BLAST search of the (then) incomplete genome database (toxodb.org) with the predicted *Plasmodium falciparum* sphingomyelin synthases (*Pf* SMS 1 and *Pf* SMS 2 - PFF1210w and PFF1215w; Huitema *et al*., 2004) identified one hypothetical protein in *T. gondii* representing a candidate functional orthologue. *Tg* SLS (50.m03113 chromosome 14) had a predicted molecular weight of 47 kDa and demonstrated 28% identity, 47% similarity to

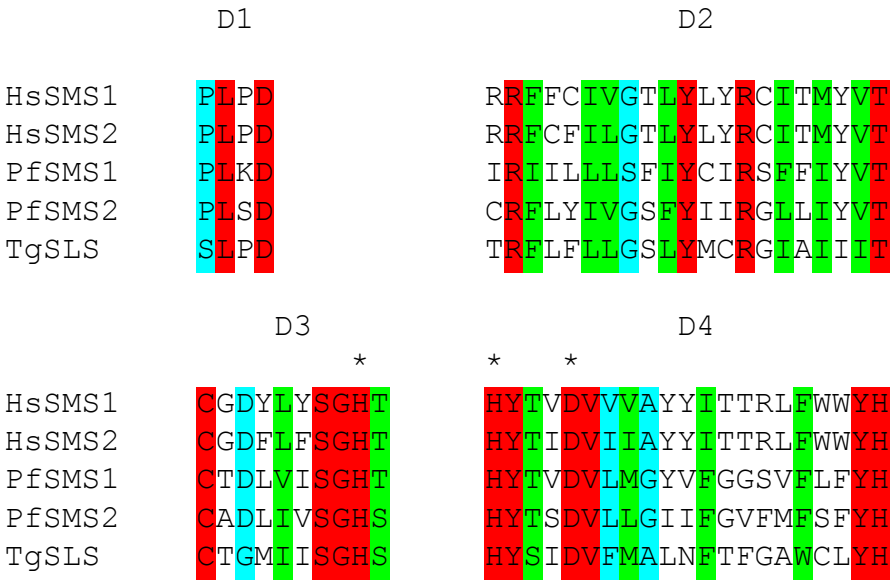
Pf SMS 1 and 27% identity, 55% similarity to *Pf* SMS 2. BLAST analysis of *Tg* SLS against the NCBI protein database showed that the hypothetical protein had 23% identity and 38% similarity to the human SMS 1 (Huitema *et al*., 2004). Subsequently, based on primary sequence analyses, 50.m03164 was also proposed as the *Tg* SLS by Welti *et al*., (2007). The predicted amino acid sequence of this predicted protein indicates that it possess multiple transmembrane domains (Figure 5-1).

Figure 5-1: Predicted protein sequence of hypothetical protein 50.m03113 (*Tg* SLS) with 8 predicted transmembrane domains (red) and the sphingolipid synthase conserved domains D 1-4 (bold underlined) (Huitema *et al*., 2004).

MPRTEMAESDSKGLPAEEDQVPALWATVHSVKFYLGRFVLATLYVFTCVYLMCLCSVASD
TFFDPRTQK**SLPD**RIHDQMLDSKPLFFATPFVVDAVTVGIIIVTIFRHAAFLKVPLNLLV
G**TR**FLFLLGSLYMCRGIAIIIT**TVP**PSRRNCVPPPVMSVGSFFYLGVLQMFSMRNE**CTGM**
IISGHSTITCCCMATWLLYGNANREDQDTKGVLFYEVARFLATRLQKVRASARQLTTQYA
ITPAFDVEEGSETVPQAESEEELSSASGVFVQEDRRKTRVAAFLRLNLLRSLCLVVGVVN
LCLIVCSFN**HYS**IDVFMALNFTFGAWCLYHCILSLIWAEKEERDKRQKVALLRSEGIAAG
AATDSDADALSEASTVSSLESGALVTGSTVKKAEPPNPPKENKPGFSAINFPLVRIGVSI
VRAIEGL

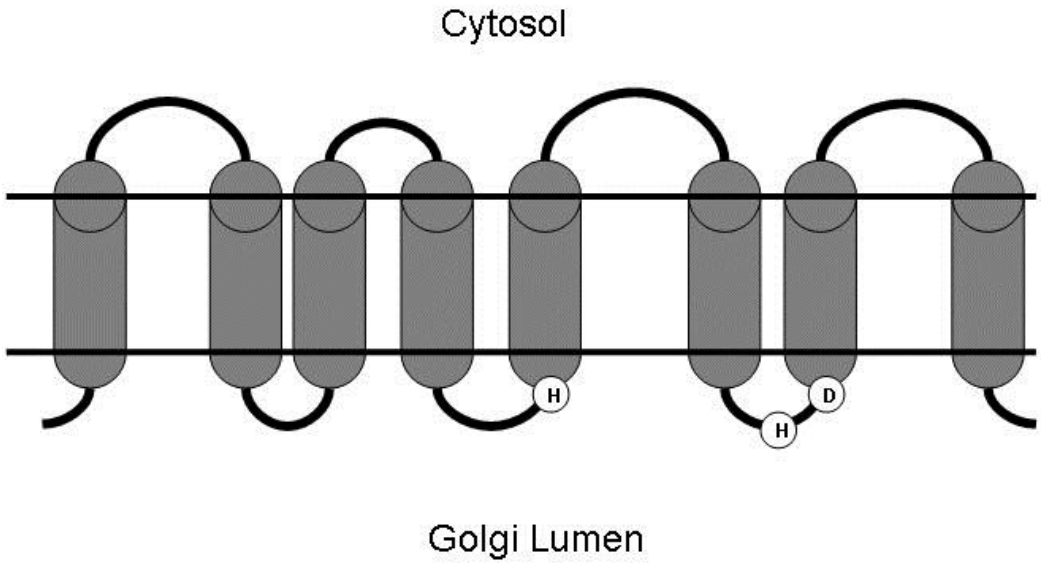
Further analyses of the conserved domains demonstrated the conservation of D1-4 in *Tg* SLS (Figure 5-2). Within D3 and D4 are the 3 residues (2 histidines, H and an aspartate, D) defining the catalytic triad that are conserved in evolution and essential for function (Huitema *et al*., 2004; Denny *et al*., 2006).

Figure 5-2: Protein sequence alignment of D1, D2, D3 and D4 from *Hs* SMS 1 and 2, *Pf* SMS 1 and 2 and *Tg* SLS. The residues highlighted in red are identical; those in green strongly similar; in blue weakly similar. The 3 residues of the predicted catalytic triad within D3 and D4 are designated by *****.



The predicted topology of *Tg* SLS is shown in Figure 5-3. Like the *Leishmania*, human and other sphingolipid synthases it is a transmembrane protein (Denny *et al*., 2006; Huitema *et al*., 2004). However, *Tg* SLS is predicted to possess 8 rather than 6 transmembrane domains. Despite this difference the catalytic triad (2 H, 1 D) is arranged at one membrane face as in related enzymes. The figure shows the arrangement predicted at the Golgi.

Figure 5-3: Predicted membrane topology of the *Tg* SLS at the Golgi membrane with the catalytic triad oriented towards the Golgi lumen.

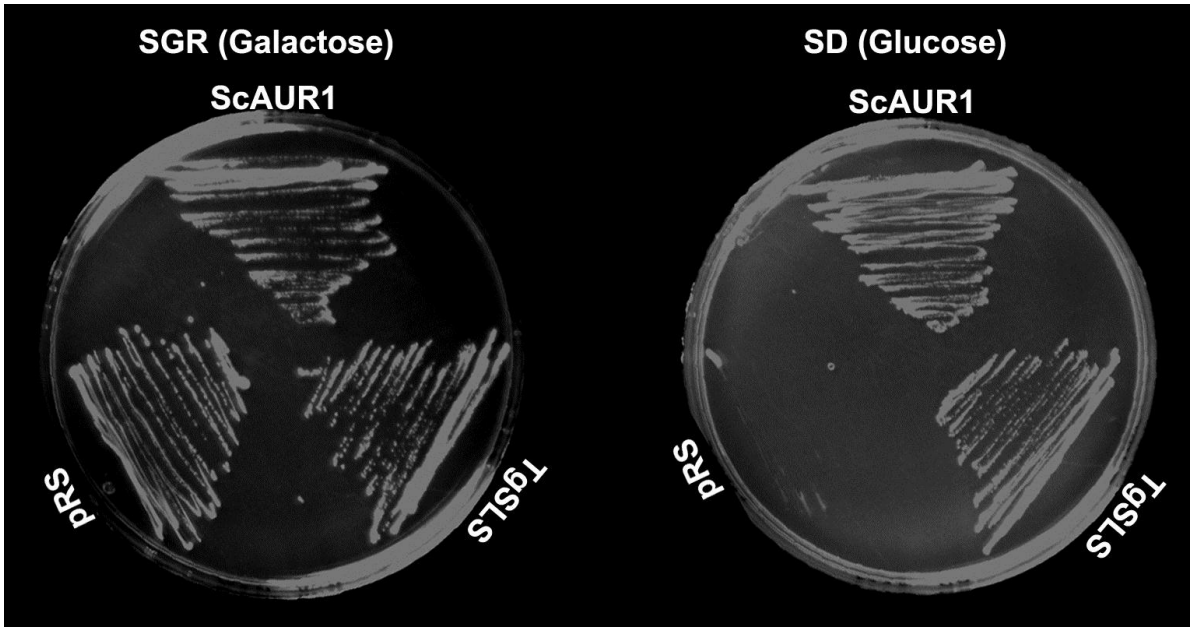


5.1.2 Tg SLS is a functional orthologue of the S. cerivisiae AUR1

Previous work in the laboratory had constructed and used an engineered yeast strain (YPH499-HIS-GAL-AUR1) with the essential IPC synthase (AUR1p) under the control of a galactose promotor (Denny *et al*., 2006). In the presence of galactose as the carbon source the yeast could express AUR1 and grow. However, with glucose AUR1 expression was suppressed and the yeasts were non-viable unless complemented by AUR1 or a functional orthologue. This approach allowed the identification of the *Leishmania major* IPC synthase (Denny *et al*., 2006). Using this approach the ability of *Tg* SLS to complement this line was established.

Since *Tg* SLS did not have introns in its coding sequence the open reading frame was amplified by PCR directly from genomic DNA and cloned into the yeast expression vector pRS426MET (URA) creating pRS426*Tg* SLS. This vector was transformed into YPH499-HIS-GAL-AUR1 and clones selected by their ability to grow in the absence of uracil and the presence of glucose (Figure 5-4).

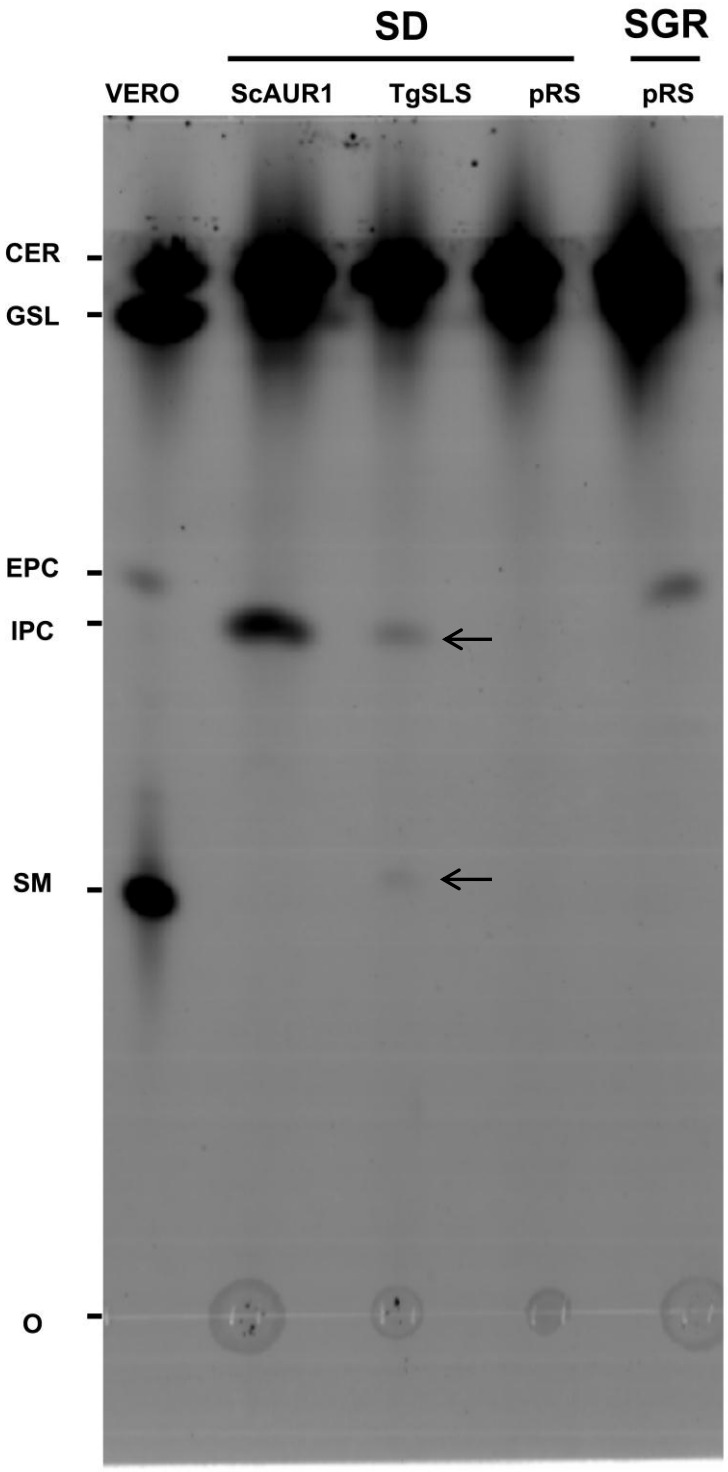
Figure 5-4: Successful complementation of YPH499-HIS-GAL-AUR1 by pRS426*Tg* SLS. Growth of the yeast is supported on SGR (galactose as carbon source) with pRS426*Tg* SLS (*Tg* SLS), a positive control – pRS426*Sc* AUR1 (*Sc* AUR1) and a negative control – empty pRS426 (pRS). In contrast only pRS426*Tg* SLS and the positive control could grow on SD (glucose as carbon source).



This complementation indicated that *Tg* SLS is a functional orthologue of AUR1.

To begin to establish the role of the AUR1 functional orthologue *Tg* SLS, the complemented YPH499-HIS-GAL-AUR1 mutant yeast was metabolically labelled with NBD C_6 ceramide when grown in SD (non-permissive media; Figure 5-5).

Figure 5-5: Separation by HPTLC of lipids extracted from YPH499-HIS-GAL-AUR1 yeast transformed with various plasmids following metabolic labelling using NBD C_6 ceramide with either glucose (SD) or galactose (SGR) as carbon sources. The positive control, YPH499-HIS-GAL-AUR1 pRS426 *Sc* AUR1 synthesized IPC as expected when grown in non-permissive SD. In contrast, the mutant complemented by *Tg* SLS synthesized a product that migrates with IPC and a second, minor, product migrating with SM. The negative control of the mutant containing empty vector pRS426 synthesized no products when incubated in non-permissive SD, but IPC synthase activity was restored in permissive SGR. Similarly labelled mammalian VERO cells served as markers for GSL, a putative EPC and SM. O is the origin, ceramide (CER) is at the front.



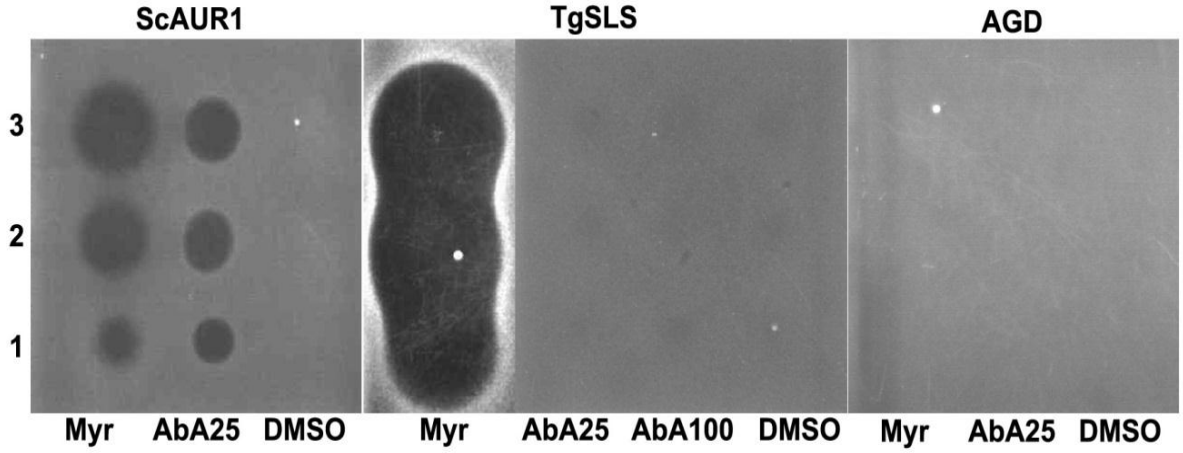
This indicated that *Tg* SLS supports the synthesis of IPC correlating with its status as a functional orthologue of AUR1**.** However, it also catalysed the synthesis of a product co-migrating with SM, supporting previous evidence for the synthesis of this species in *T. gondii* (Azzouz *et al*., 2002). There is no evidence that *Tg* SLS drives the formation of

EPC, which has been identified as a major phosphosphingolipid in the parasite (Welti *et al*., 2007).

The presence of IPC has previously been proposed on the basis on metabolic labelling with tritiated inositol and analyses of synthesized lipid species following mild alkaline treatment (Sonda *et al*., 2005). In this same study, electron microscopy analyses of *T. gondii*-infected HFF treated with 10 μg/ ml of aureobasidin A (Ab A) for 24 hours showed severe alterations in the parasite-containing vacuoles, with an increase in *T. gondii* vacuolization and a loss of intracellular structures leading to cell death (Sonda *et al*., 2005). Ab A is a potent antibiotic active against a variety of pathogenic fungi shown to inhibit IPC synthase.

Based on the anti-protozoal effect of Ab A Sonda *et al*., (2005) proposed a putative *T. gondii* IPC synthase as the Ab A target. Having isolated a functional orthologue of the *S. cerevisae* AUR1 the sensitivity of *Tg* SLS was analysed by diffusion assay (Figure 5-6). The sphingolipd bypass mutant AGD was used as a negative control. AGD is able to grow without synthesizing sphingolipids and therefore is able to tolerate loss-of-function mutations in both serine palmitoyltransferase and IPC synthase (Nagiec *et al*., 1997). Myriocin is an inhibitor of SPT mediating the first step in sphingolipid biosynthesis, Ab A inhibits the IPC synthase of *S. cerevisiae* and, perhaps, *T. gondii* (Sonda *et al*., 2005).

Figure 5-6: Agar diffusion assays of YPH499-HIS-GAL-AUR1 complemented with *Sc* AUR1 or *Tg* SLS. AGD yeast lacking functional SPT and AUR1 was the negative control. Volumes of 1, 2 and 3µl of Myriocin, aureobasidin A and DMSO spotted on to yeast plates. As expected, *Sc* AUR1 was sensitive to myriocin (Myr) and aureobasidin A at 25µM and 100µM (Ab A25 and Ab A100) whilst the AGD was insensitive to both inhibitors. Interestingly, *Tg* SLS was resistant to Ab A at both concentrations but was hyper-sensitive to myriocin as evidenced by large zones of exclusion.

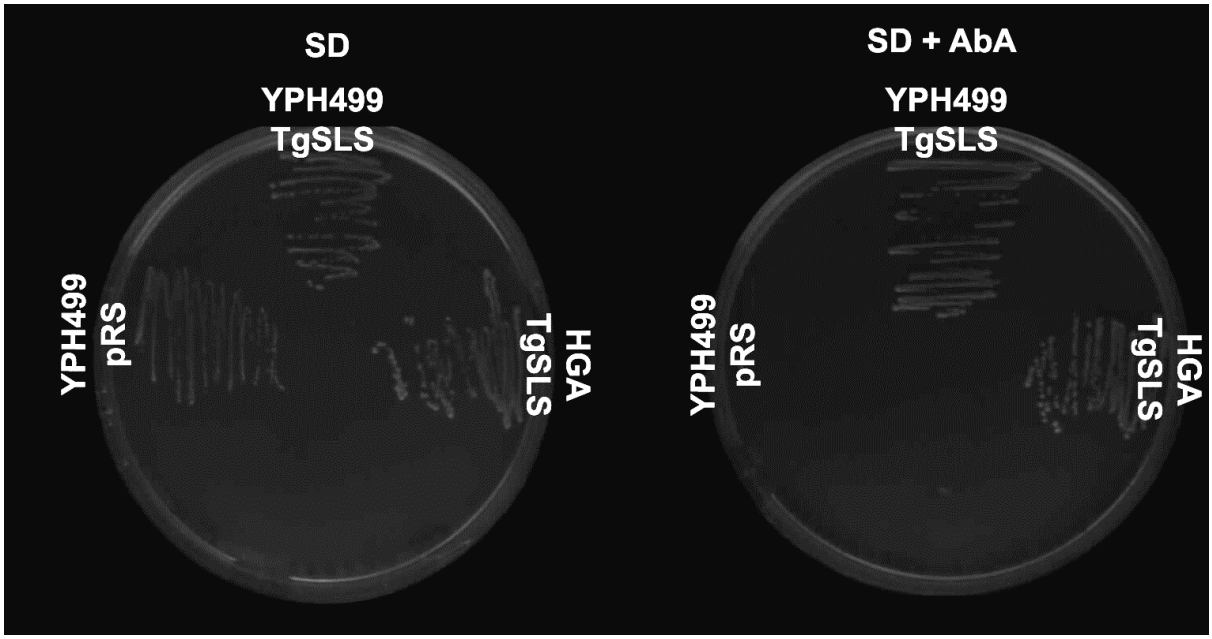


These results showed that *Tg* SLS conferred Ab A resistance to the yeast. The hyper-sensitivity to myriocin resembled that seen in the same mutant yeast complemented by the *Leishmania major* IPC synthase (Denny *et al*., 2006). The reasons for this are unknown but perhaps reflect a suboptimum functionality of the protozoan sphingolipid synthases in the yeast making the complemented lines more sensitive to upstream inhibition of sphingolipid biosynthesis (myriocin and SPT).

To confirm the Ab A resistance properties of *Tg* SLS, pRS426*Tg* SLS was transformed into wild type yeast, YPH499, and was empty pRS426 as a control. The growth of these

was analysed on SD-URA with or without Ab A. YPH499-HIS-GAL-AUR1 pRS426*Tg* SLS was also analysed as a further control (Figure 5-7).

Figure 5-7: YPH499 transformed with pRS426*Tg* SLS or empty pRS426 and YPH499-HIS-GAL-AUR1 (HGA) pRS426*Tg* SLS streaked onto SD-URA with or without 4.5 µM Aureobasidin A (Ab A). *Tg* SLS confers Ab A resistance to YPH499.

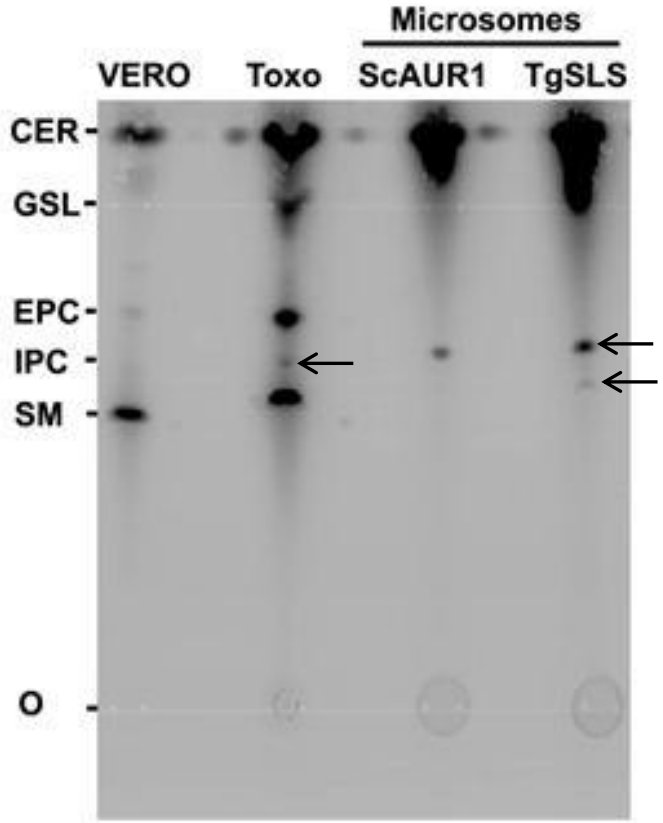


Together the data in Figures 5-6 and 5-7 clearly indicate that *Tg* SLS is insensitive to Ab A suggesting that the anti-protozoal effects observed with this drug (Sonda *et al*., 2005) are not due to inhibition of this enzyme.

5.2 BIOCHEMICAL ANALYSES OF *TG* SLS

Given the observation that *Tg* SLS appears to support the synthesis of 2 products (IPC and SM) biochemical analyses were necessary to fully establish its functionality. Utilising the *Tg* SLS complemented yeast microsomes were isolated and used in biochemical assays as described in Materials and Methodology. Incubating the microsomes with FL C_5 ceramide as described lead to the formation of complex lipid products that could be analysed after fractionation by HPTLC. FL C_5 ceramide was used in this experiment due to its stability relative to NBD C_6 ceramide and the enhanced labelling of *T. gondii* parasites seen as a result (Figure 5-8).

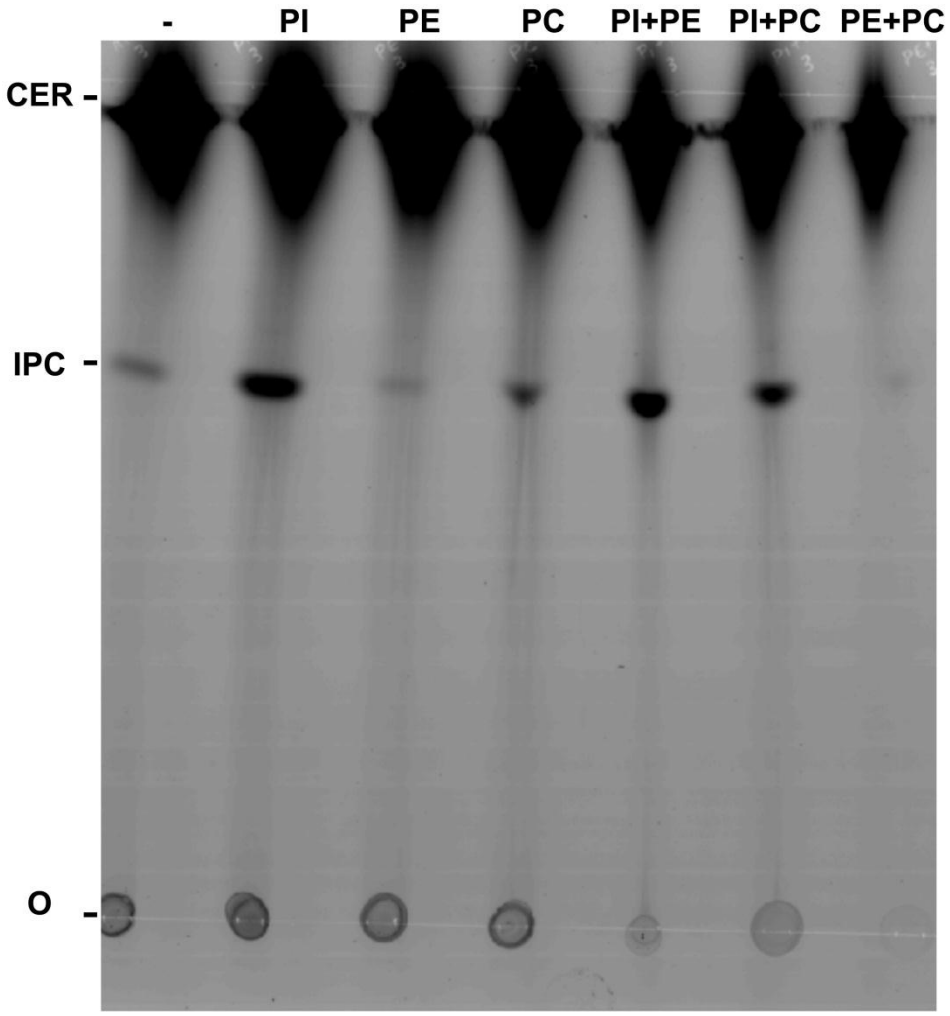
Figure 5-8: HPTLC separation of lipids after incubation of isolated microsomes, whole parasites (Toxo) and mammalian (VERO) cells with FL C_5 ceramide. Microsomal material from *Sc* AUR1 yeast produced IPC as expected, *Tg* SLS microsomes lead to the formation of one product migrating with IPC and another with SM (arrows). This was in agreement with the metabolic labelling in Figure 5.5. Isolated metabolically labelled *T. gondii* (Toxo) produced 3 major complex sphingolipid products predicted to be GSL, EPC and SM. This result was in agreement with previous analyses (Azzouz *et al*., 2002; Welti *et al*., 2007). However, a fourth minor product was also apparent co-migrating with IPC (arrow). O is the origin, ceramide (CER) is at the front.



These data support those in Figure 5-5 and indicate that *Tg* SLS is a dual function enzyme catalysing the production of both IPC and SM. In addition, the synthesis of the phosphosphingolipids SM, EPC and IPC is indicated in isolated, intact *T. gondii* parasites. Whilst *Tg* SLS may be involved in the synthesis of SM and IPC there was no evidence of it having a role in EPC production.

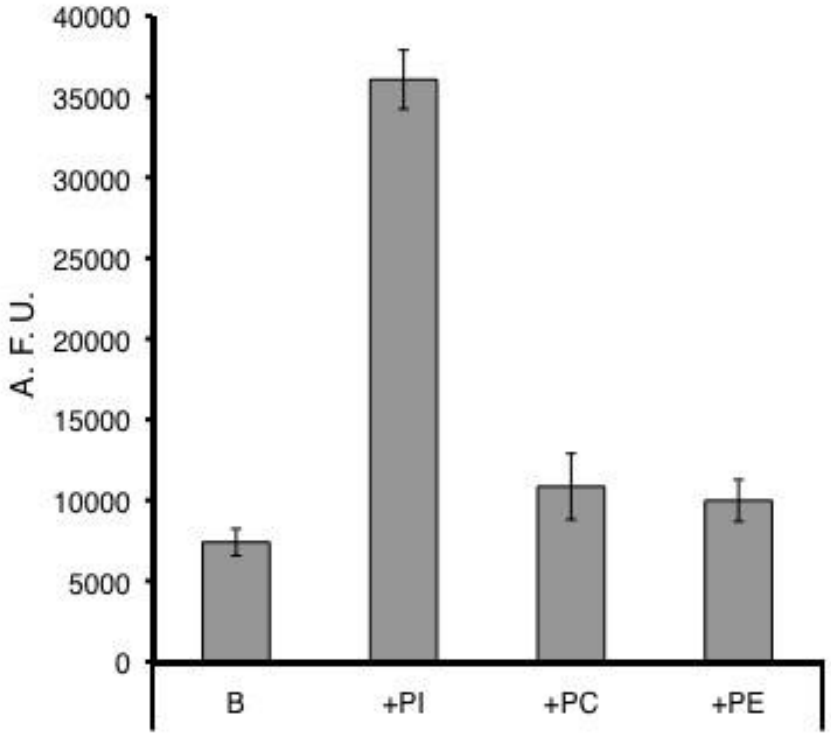
To further understand this dual functionality the microsomes were washed with CHAPS to reduce or remove contaminating substrate associated with microsomal membranes, in this case ceramide, phosphatidylinositol (PI) and phosphatidylcholine (PC). CHAPS is a zwitterionic detergent used to solubilize biological macromolecules such as proteins (Garner, Smith and Hoper, 2007). It is used as a non-denaturing solvent in the process of protein purification and is especially useful in purifying membrane proteins, such as *Tg* SLS, which are often sparingly soluble or insoluble in aqueous solution due to their native hydrophobic cellular environment (Aeed *et al*., 2004). The washed microsomal fractions could then be reacted with different donor substrates (PI, PE or PC) singly or in combination and fluorescent NBD C_6 ceramide, the acceptor substrate (Figure 5-9).

Figure 5-9: HPTLC separation of lipids after reaction of CHAPS-washed microsomes with NBD C_6 ceramide and either no donor substrate (-) or PI, PE or PC, or combinations of these. Only the addition of PI led to increase in the production of a product, this migrated with IPC. The addition of PE and PC did not lead to any significant increase in product formation. The addition of PE and PC in combination or with PI has no additional effect. PI - phosphatidyl inositol, PE - phosphatidyl ethanolamine, PC - phosphatidyl choline, O - origin of solvent front, ceramide (CER) is at the front.



These results indicate that *Tg* SLS only functions as an IPC synthase in this assay since only PI is functional as a donor substrate. In addition, unlike the similarly functional *Trypanosoma brucei* orthologue (Chapter 6; Mina *et al*., 2009) neither PE nor PC were significantly inhibitory (as quantified after scanning) suggesting they are not competitive binders. Intriguingly, the addition of PC alone lead to a small apparent increase in IPC production. To investigate further the experiment was repeated 3 times. The fluorescence intensity of IPC was quantified with each of the 3 donor substrates (Figure 5-10).

Figure 5-10: IPC produced in *in vitro* assay with no donor substrate (B), phosphatidylinositol (PI), phosphatidylethanolamine (PE) or phosphatidylcholine (PC). AFU – Arbitrary Fluorescence Units). Mean of 3 independent experiments, standard deviation indicated.

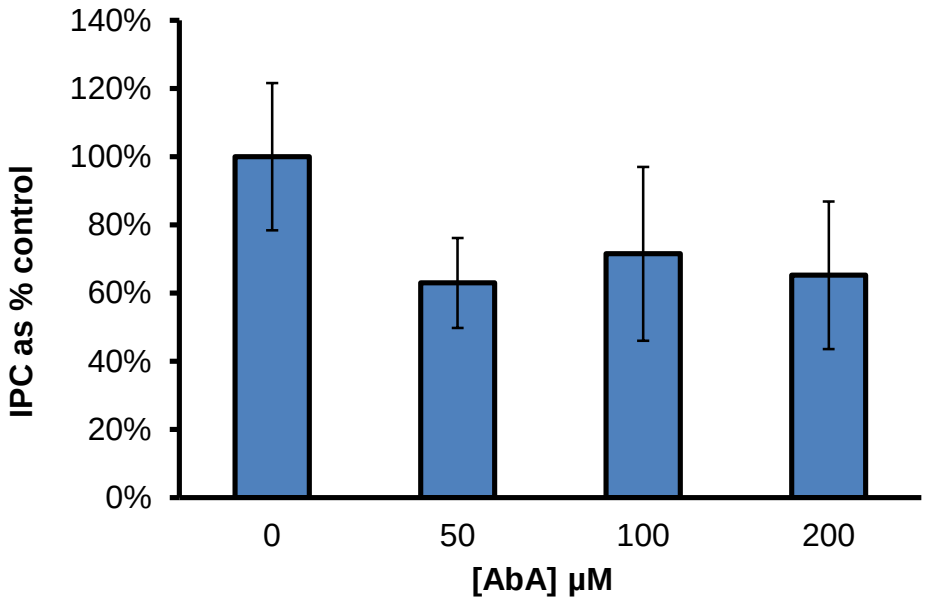


These data revealed no apparent increase in the production of IPC (or any other products) apart from on the addition of PI as donor substrate. This confirmed that *Tg* SLS functions as an IPC synthase. However, given the observation that *Tg* SLS appears to catalyse the formation of SM (Figures 5-5 and 5-8), the failure of the enzyme to utilise PC as donor substrate is surprising. All donor substrates employed were from bovine liver extracts, mixed natural product for PC the predominant species is C36:2. In contrast, *S. cerevisiae*, where *Tg* SLS appears to function as an SM synthases, possesses predominantly C32:2 and C34:2 PC (Boumann *et al*., 2003). To investigate whether these shorter lipids are accepted preferentially by *Tg* SLS, C28:2, C30:2 and C32:2 PC were assayed. None of these functioned as donor substrates for SM production (data not shown) and any SM synthase

role of the enzyme remains unclear.

To further analyse the resistance of *Tg* SLS to Ab A inferred from cellular assays (Figure 5-6 and 5-7) CHAPS-washed microsomes with PI were pre-incubated with a series of concentrations of Ab A (0 - 200 µM) before the addition of acceptor substrate, NBD C_6 ceramide (Figure 5-11).

Figure 5-11: Inhibitory effect of aureobasidin A (Ab A) on *Tg* SLS assayed *in vitro*. Fluorescence intensity of IPC was established following fractionation by HPTLC and normalised with respect to a control (0 µM Ab A). Mean of 3 independent experiments, standard deviation indicated.



IPC production was diminished by approximately 40% in the presence of 50 µM Ab A. However, there was no significant impact when the concentration of Ab A was increased in the assay. Correlating with cellular assays *Tg* SLS is relatively resistant to the potent fungal IPC synthase inhibitor, Ab A. This demonstrates that *Tg* SLS, and by extension IPC synthesis, is not the target of Ab A in *T. gondii* as previously proposed (Sonda *et al.*,

2005). It is possible that the effects of Ab A against *T. gondii* noted by Sonda *et al*., (2005) were due to other, non-specific, modes of actions as previously noted for the kinetoplastid intracellular pathogen, *T. cruzi* (Figueiredo *et al*., 2005; Salto *et al*., 2003).

5.3 CONFIRMATION OF THE PRESENCE OF IPC IN *T. GONDII* TACHYZOITES

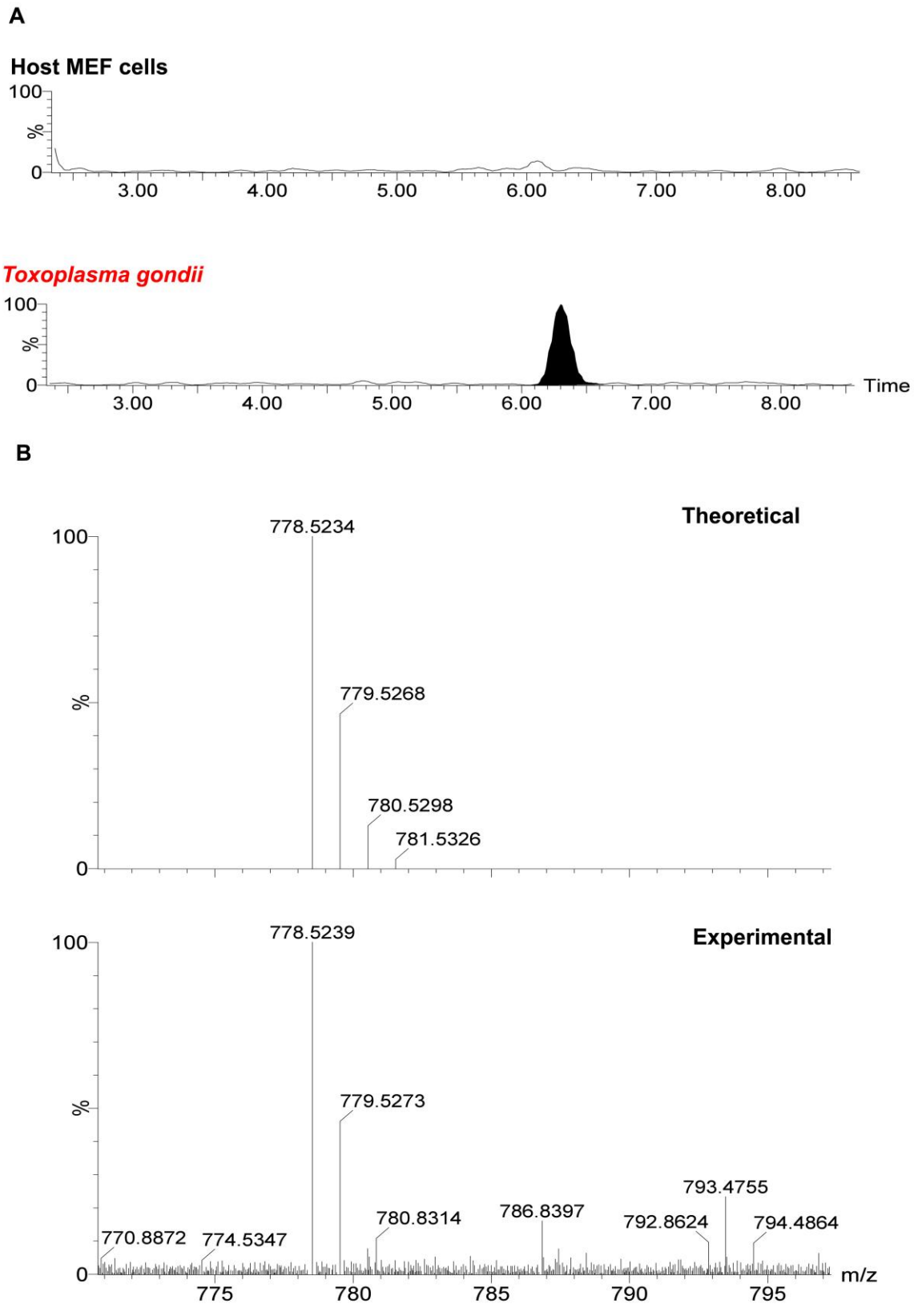
As mentioned above metabolic labelling of *T. gondii* tachyzoites with tritiated inositol, followed by analysis the lipids synthesized by HPTLC, revealed the presence of a lipid proposed to be IPC (Sonda *et al*., 2005). The results above identify the *Tg* SLS and confirm it functions as an IPC synthase. In addition, FL C_5 ceramide labelling of *T. gondii* tachyzoites demonstrated the presence of a synthesized sphingolipid co-migrating with IPC (Figure 5-8). However, the metabolic labelling studies described here and previously do not provide definitive evidence of the presence of IPC in *T. gondii* parasites. In order to confirm the presence of this parasite specific lipid species further analyses are required using mass spectrometry. These studies were carried out in collaboration with Dr Sabrina Sonda (Zurich) and Dr Josefina Casas (Barcelona) by liquid chromatography-mass spectrometry (LC-MS) analysis (Munoz-Olaya *et al*., 2008) of lipids extracted from *T. gondii* (RH strain) and host cells (MEF).

Briefly, *Toxoplasma gondii* were harvested from infected MEF host cells by passage through a 26-gauge needle and purified by separation on Sephadex-G25 columns (Amersham). Sphingolipid extracts were prepared as described (Merrill *et al*., 2005) and analysed using the Waters Aquity UPLC (Ultra Performance Liquid Chromatography) system connected to a Waters LCT Premier orthogonal accelerated time of flight mass spectrometer (Waters, Millford, MA) (Bottova *et al*., 2009) operated in negative electrospray ionization to detect IPC (Denny *et al*., 2004). Positive identification of IPC

was based on the accurate mass measurement with an error <5 ppm and its LC retention time (Bottova *et al*., 2009). The resultant spectra are shown in Figure 5-12.

Figure 5-12: (A). Analyses of fractions from UPLC column, x-axis is time in minutes that fractions are eluted from the column. Fractions containing mass of 778.523, mass of hypothetical IPC, were detected in the negative ion spectra. Whilst the *T. gondii* lipids demonstrated a fraction with this mass, the same fraction from the mammalian MEF host cells lacks this species.

(B). Mass spectrometry analyses of this fraction showed a good match with the hypothetical species expected from IPC. 778.5239 and a protonated species, 779.5273. The elemental composition report strongly suggested that this represented IPC: formula C40 H77 N O11 P, IPC C18:1/C16:0.

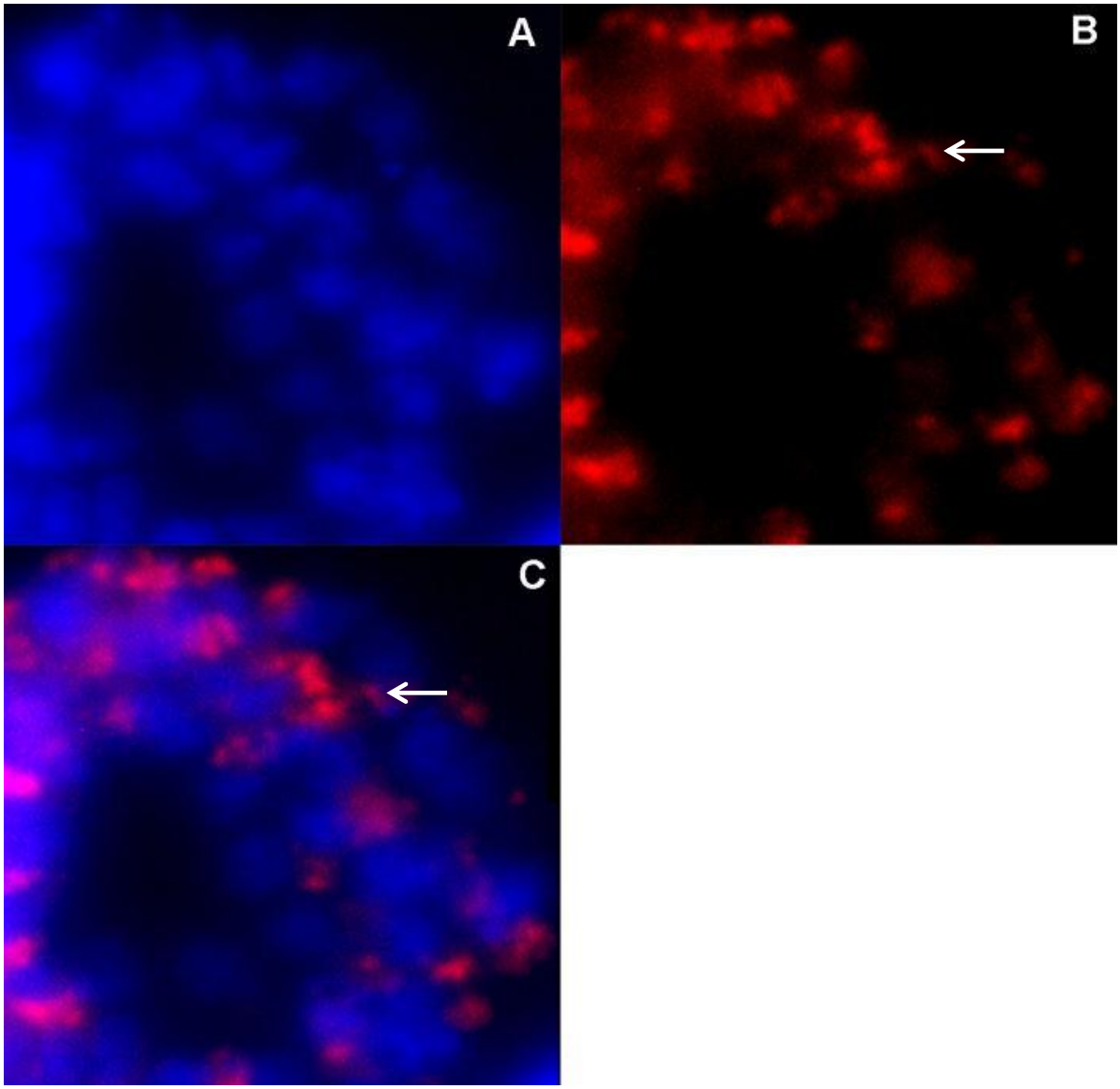

A
Host MEF cells
100
%
0
3.00
4.00
5.00
6.00
7.00
8.00
Toxoplasma gondii
100
%
0
3.00
4.00
5.00
6.00
7.00
8.00
Time
B
Theoretical
778.5234
779.5268
780.5298
781.5326
100
%
0
Experimental
778.5239
779.5273
793.4755
780.8314
786.8397
792.8624
794.4864
770.8872
774.5347
100
%
0
775
780
785
790
795
m/z

These data confirm the presence of IPC in *T. gondii* tachyzoites and correlate with the identification of *Tg* SLS, an enzyme which functions as an Ab A resistant IPC synthase. A previous lipidomic analysis of *T. gondii* tachyzoites had failed to identify IPC (Welti *et al*., 2007). Notably, this study did not fractionation the material prior to mass spectrometry analyses perhaps leading to minor species being undetected.

5.4 THE SUBCELLULAR LOCALISATION OF *T. GONDII*

The Golgi apparatus is regarded as the primary site for phosphosphingolipid synthesis and, for example, human SM synthase has been localised here (Huitema *et al*., 2004). To begin to establish the localisation of *Tg* SLS in *T. gondii* tachyzoites the open reading frame was cloned into the expression vector pT8 with a C-terminal TY epitope tag (Appendix III). Transient transfection of this plasmid into *T. gondii* as described in Materials and Methodology, allowed the localisation of *Tg* SLS using a specific monoclonal antibody provided by Prof Keith Gull, Oxford (Figure 5-13).

Figure 5-13: Epi-fluorescent microscopy image of *Tg* SLS-TY stained with a specific monoclonal antibody (B, red). 4', 6-diamidino-2-phenylindole (DAPI) stain was used to stain the nuclei to visualise the parasites (A, blue). C- overlay of images of A and B showing the tachyzoite nuclei and the localisation of *Tg* SLS-TY into distinct foci (arrow).



The distinct structures in which *Tg* SLS resides may represent the Golgi apparatus. However, further analyses (co-localisation with known markers and / or immuno-electron microscopy) will be required to confirm this.

SUMMARY

Tg SLS was identified as an AUR1 functional orthologue conferring Ab A resistance to *S. cerevisiae.* However, unlike AUR1, *Tg* SLS was able to produce 2 complex sphingolipids: a major species co-migrating with IPC and a minor species co-migrating with SM. Biochemical analyses confirmed that the enzyme functions as a true IPC synthase and that this action is Ab A resistant. However, SM synthase (or EPC synthase activity) could not be constituted *in vitro.* These 2 species had previously been identified as major *T. gondii* phosphosphingolipids in lipidomic analyses (Welti *et al*., 2007). The presence of IPC has only been inferred from metabolic labelling studies (Sonda *et al*., 2005). LC-MS analyses in this study have confirmed the presence of IPC in the parasite, although perhaps as only a minor species. Therefore, the *Tg* SLS is proposed to function as an IPC synthase *in vivo*, with this activity likely to be localised to the Golgi apparatus.

CHAPTER 06

OTHER SPHINGOLIPID SYNTHASES

6 OTHER SPHINGOLIPID SYNTHASES

6.1 MAMMALIAN SPHINGOMYELIN SYNTHASES (*HS* SMS)

The predominant sphingolipid in mammals is sphingomyelin (SM) which is formed by the transfer of phosphocholine from the donor substrate phosphatidylcholine (PC) to the acceptor substrate ceramide. The enzyme responsible for this is SM synthase which has 2 isoforms in human cells, *Hs* SMS 1 and *Hs* SMS 2 (Huitema *et al*., 2004). These isoforms are both transmembrane proteins maintaining the four evolutionarily conserved domains, D1-4, and the catalytic triad essential for functionality (Figure 6-1, *Hs* SMS 1 as an example). However, whereas *Hs* SMS 1 is Golgi localized, *Hs* SMS 2 is found at the plasma membrane. Neither these enzymes were demonstrated to function as IPC synthases (Huitema *et al*., 2004).

Figure 6-1: Protein coding sequence of *Hs* SMS 1 with 6 transmembrane domains (red underlined), active site residues (bold) and conserved domains (D1-4 grey highlight). *Hs* SMS 2 shares this structure.

MKEVVYWSPKKVADWLLENAMPEYCEPLEHFTGQDLINLTQEDFKKPPLCRVSSDNGQ

RLLDMIETLKMEHHLEAHKNGHANGHLNIGVDIPTPDGSFSIKIKPNGMPNGYRKEMI

D1

KIPMPELERSQYPMEWGKTFLAFLYALSCFVLTTVMISVVHERVPPKEVQPPLPDTFF

D2

DHFNRVQWAFSICEINGMILVGLWLIQWLLLKYKSIISRRFFCIVGTLYLYRCITMYV

D3

VTTLPVPGMHFNCSPKLFGDWEAQLRRIMKLIAGGGLSITGSHNMCGDYLYSG**H**TVML

D4

TLTYLFIKEYSPRRLWWYHWICWLLSVVGIFCILLAHD**H**YTV**D**VVVAYYITTRLFWWY

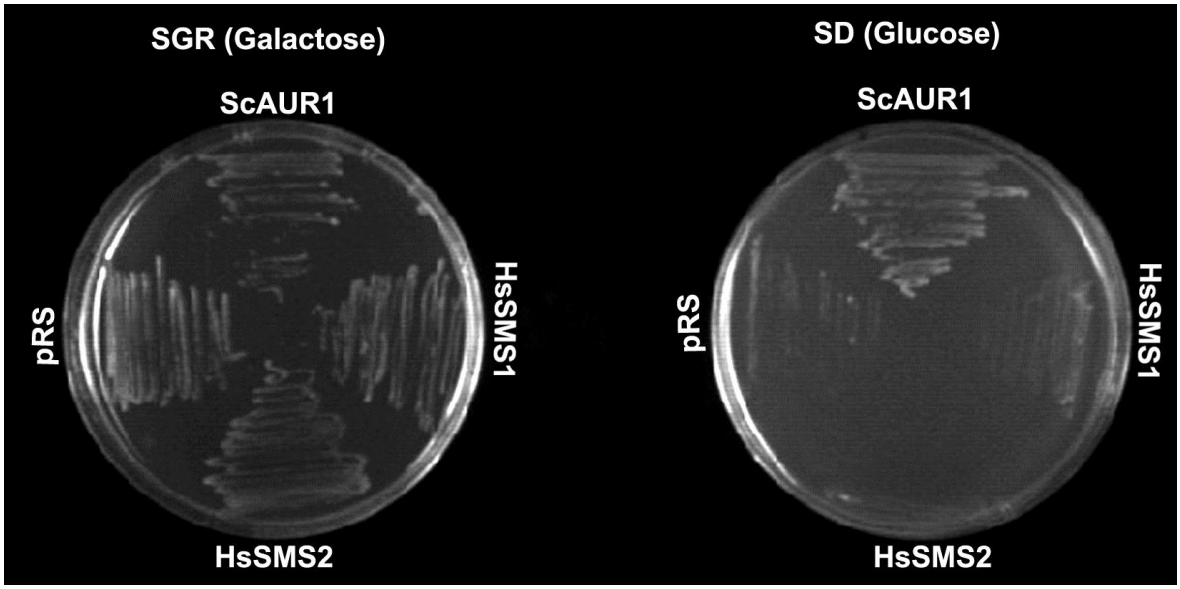
HTMANQQVLKEASQMNLLARVWWYRPFQYFEKNVQGIVPRSYHWPFPWPVVHLSRQVK

YSRLVNDT

6.2 DO *HS* SMS 1 OR *HS* SMS 2 RESCUE THE YEAST AUR1 MUTANT?

The *Hs* SMS 1 and *Hs* SMS 2 open reading frames were amplified from cDNA cloned (Dr Joost Holthuis, Utrecht) and ligated into the pRS426MET yeast expression vector. However, on transformation neither pRS426*Hs* SMS 1 nor pRS426*Hs* SMS 2 complemented the YPH499-HIS-GAL-AUR1 yeast (Denny *et al*., 2006) indicating that SM synthase activity alone is not sufficient to rescue the mutant (Figure 6-2). As described in Chapter 3, in YPH499-HIS-GAL-AUR1 yeast the essential IPC synthase (AUR1) is under the control of a galactose promotor and, therefore, will not grow in the presence of the suppressor glucose. This auxotrophic mutation can be overcome by complementation as shown in Chapter 3.

Figure 6-2: YPH499-HIS-GAL-AUR1 transformed with pRS426 *Hs* SMS 1 and pRS426 *Hs* SMS 2 and grown on permissive (SGR, Galactose) and non-permissive (SD, Glucose) media. *Hs* SMS 1 and *Hs* SMS 2 transformed yeast only grew in the presence of galactose, as did yeast transformed with empty vector (pRS). In contrast *Sc* AUR 1 complemented yeast could grow on SD media (in the presence of glucose).



Although the human enzymes are known to be functional when expressed in yeast (Huitema *et al*., 2004) it is clear that expression cannot compensate for the loss of AUR1. Therefore, it is likely that IPC itself is essential for yeast survival and growth.

6.3 *TRYPANOSOMA BRUCEI* SPHINGOLIPID SYNTHASES (*Tb* SLS)

6.3.1 Identification of Tb SLS

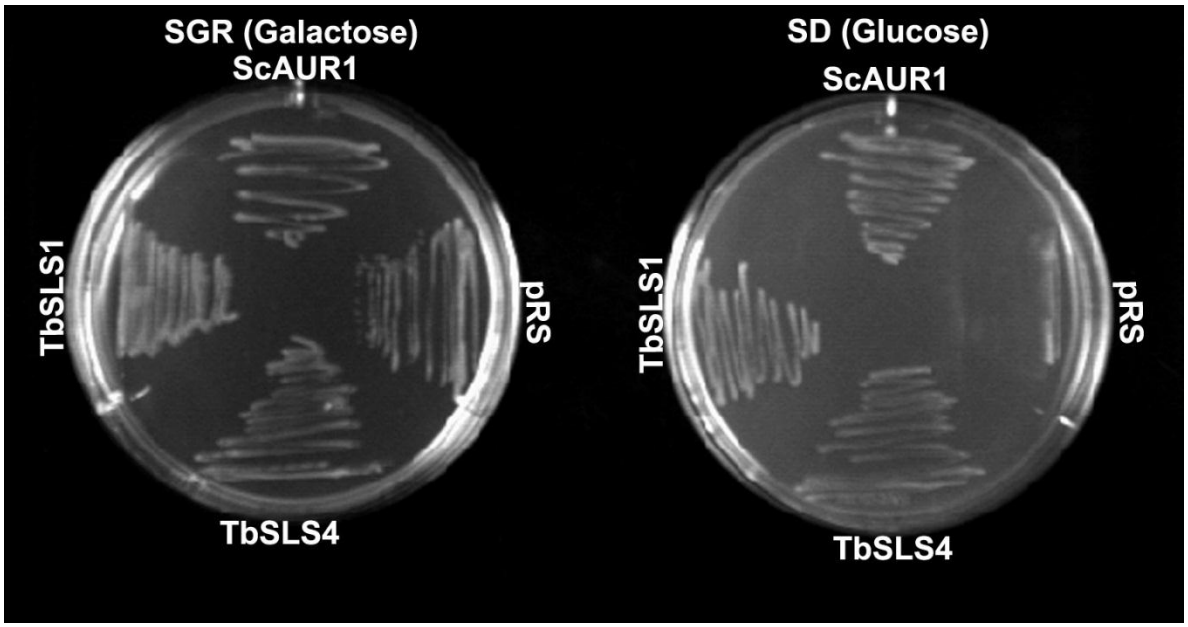
Trypanosoma brucei is an insect vector-borne kinetoplastid protozoan parasite related to the *Leishmania* species. Both of these parasites cause neglected tropical diseases (NTDs), African sleeping sickness (African trypanosomiasis) and leishmaniasis, respectively. Four gene sequences (accession numbers Tb09211.1000, Tb09.211.1010, Tb09.211.10 and Tb09.211.1030) were first identified by Denny *et al*., (2006) in the genome database (genedb.org) as sequence orthologues of the *Leishmania major* IPC synthase (*Lmj* IPCS), a putative drug target. Like *Lmj* IPCS and *Tg* SLS (Chapter 5), *Tb* SLS possess the conserved D1, D2, D3 and D4 domains first identified in the SM synthase family (Denny *et al*., 2006; Sutterwala *et al*., 2008).

One of the predicted *T. brucei* SLS isoforms (Tb09.211.1000; *Tb* SLS 4) was the focus of this study. In addition, the isoform most distant from *Tb* SLS 4 with respect to the internal variable domain (Tb09.211.1030; *Tb* SLS 1) was also subjected to preliminary analyses (Mina *et al*., 2009).

6.3.2 Tb SLS 1 and Tb SLS 4 are functional orthologues of AUR1

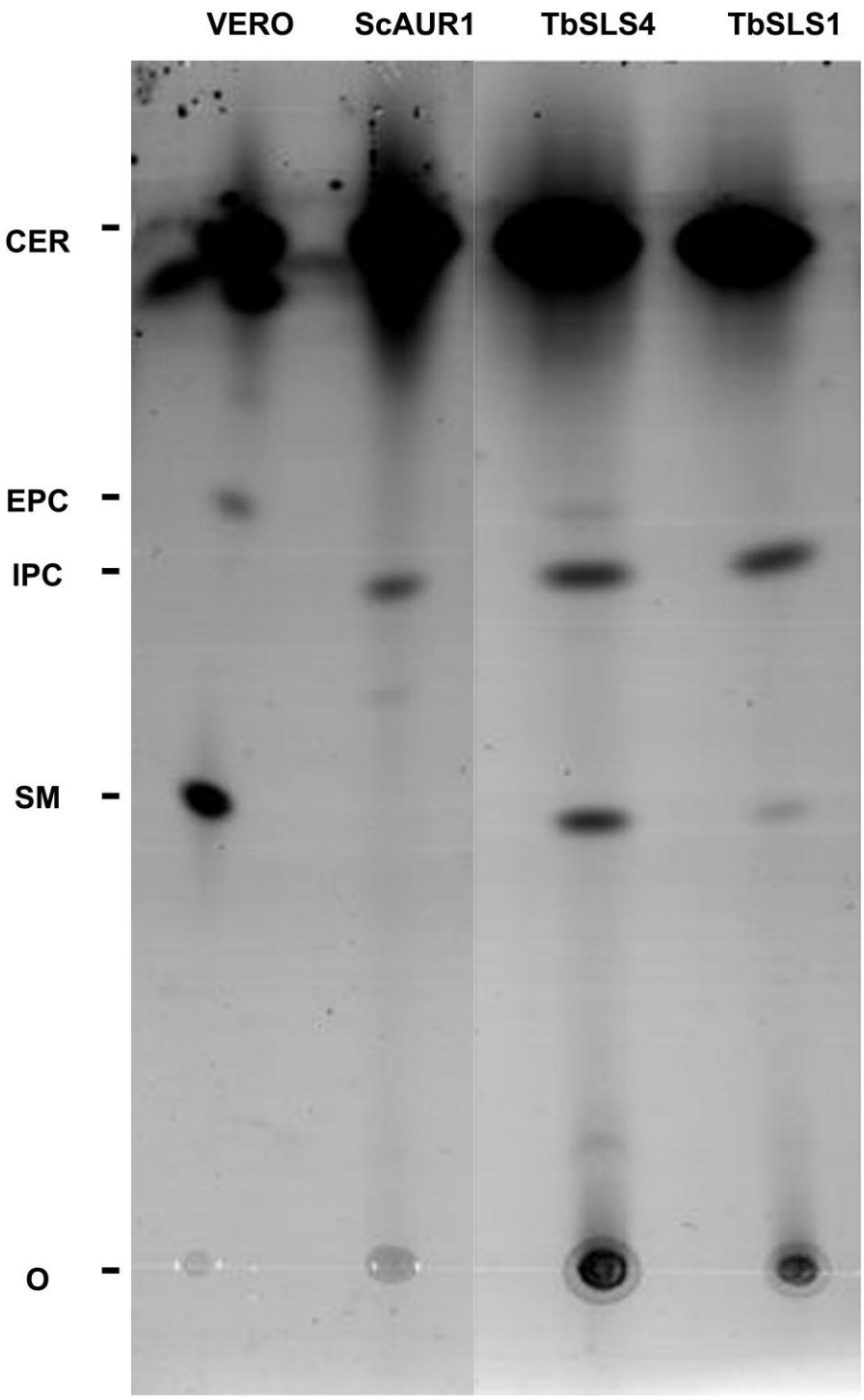
Tb SLS 1 and *Tb* SLS 4 open reading frames were amplified from genomic *T. brucei* Lister 427 and ligated into the pRS426MET yeast expression vector. Unlike the mammalian SMS isoforms above, both the *T. brucei* orthologues complemented the YPH499-HIS-GAL-AUR1 mutant yeast line indicating that they are functional orthologues of AUR1p (Figure 6-3).

Figure 6-3: Transformation with pRS426*Tb* SLS 4 and *pR*S426*Tb* SLS 1, as well as pRS426*Sc* AUR1, rescue the auxotrophic mutant YPH499-HIS-GAL-AUR1 allowing it to grow in non-permissive SD (glucose containing) media. As expected empty pRS426 did not complement the auxotrophic mutant. However, all lines are viable in the absence of glucose and the presence of galactose (SGR).



To begin to understand the full function of *Tb* SLS 1 and *Tb* SLS 4, the complemented YPH499-HIS-GAL-AUR1 mutants were metabolically labelled with NBD C_6 ceramide when grown in SD (non-permissive media; Figure 6-4).

Figure 6-4: Separation by HPTLC of lipids extracted from YPH499-HIS-GAL-AUR1 yeast transformed with various plasmids following metabolic labelling using NBD C_6 ceramide in non-permissive SD (glucose as the carbon source). As expected *Sc* AUR1 complemented yeast synthesize only labelled IPC. However, in contrast *Tb* SLS 1 and *Tb* SLS 4 synthesized two predominant sphingolipid species, one of which co-migrated with IPC, the other with SM. In addition, in *Tb* SLS 4 complemented yeast a minor labelled species was evident which co-migrated with the predicted EPC detected in labelled mammalian VERO cells. O, origin; IPC, inositol phosphorylceramide; SM, sphingomyelin; EPC, ethanolamine phosphorylceramide; CER, ceramide (migrating at the front).



Tb SLS 1 and *Tb* SLS 4 complemented YPH499-HIS-GAL-AUR1 yeast were shown to synthesize two major labelled lipid species. One of these co-migrated with SM, the other with IPC. The latter at levels equivalent to those produced in the AUR1 complemented yeast (Figure 6-4). In addition, *Tb* SLS 4 complemented yeast synthesized a third species

co-migrating with the putative EPC produced by labelled VERO cells. Notably, unlike IPC, SM is known to be present in the pathogenic blood stream form of the parasite (Patnaik *et al*., 1993). IPC is found in insect stage procyclic parasites (Guther *et al*., 2006).

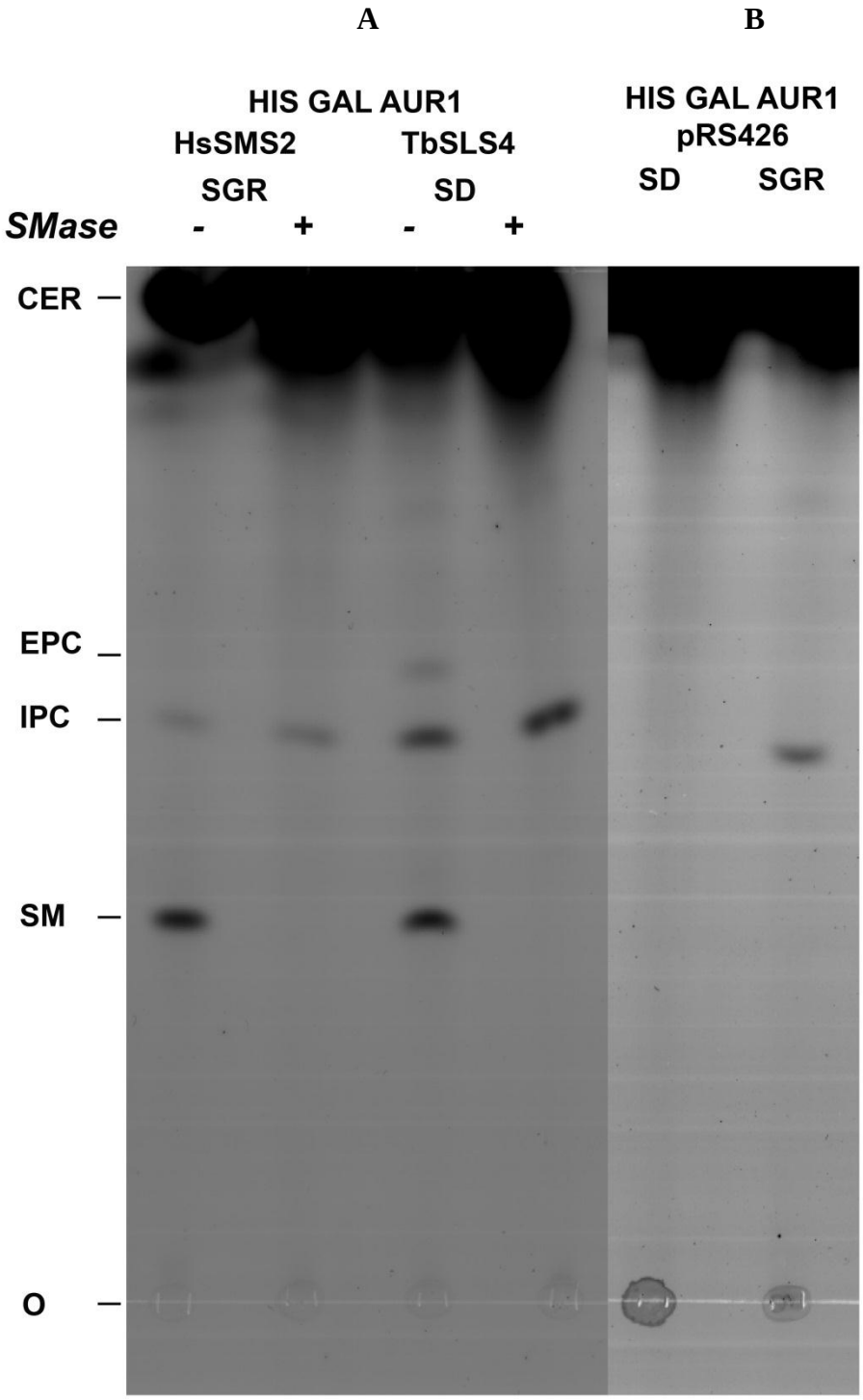
Given this lifecycle distribution *Tb* SLS 4 was chosen for further study due to the relative predominance of the SM-like species in the labelled complemented yeast. Importantly, the SM-like species proved to be susceptible to sphingomyelinase (SMase; which breaks down SM into phosphorylcholine and ceramide) when lipid extracts were treated with this enzyme, thereby confirming its identity (Figure 6-5 A). The putative EPC was also sensitive which is may be not surprising given the structural similarity between the head groups species, choline ($C_5H_{14}NO$) and ethanolamine (C_2H_7NO).

In contrast the IPC produced was insensitive to this enzyme treatment. Extracts from the auxotrophic mutant expressing *Hs* SMS 2, which produce an equivalent quantity of labelled SM under permissive conditions (SGR), acted as a control for SMase activity. As a further control YPH499-HIS-GAL-AUR1 cells harbouring an empty vector (pRS426) were cultured in both permissive (SGR) and non- permissive (SD) media. Both lines grew equivalently in both media for 16 h and for a further 8 h after dilution to an optical density 0.3 OD_{600}, and remained viable. Labelling of these dividing cells with NBD C_6 ceramide in their respective media under the same conditions as above demonstrated that in non-permissive SD no labelled IPC is produced, demonstrating the down-regulation of AUR1p (Figure 6-5 B).

Figure 6-5: (A) Sphingomyelinase (SMase) treatment of lipids extracted from YPH499-HIS-GAL-AUR1 complemented with *Tb* SLS 4, grown in SD and labelled with NBD C_6 ceramide demonstrated that the predicted SM species is SMase sensitive, as is the putative EPC. In contrast the predicted IPC is insensitive. As a control, YPH499-HIS-GAL-AUR1 expressing *Hs* SMS 2, grown in permissive galactose (SGR media) was utilized as they produce equivalent quantities of labelled SM to the *Tb* SLS 4 line.

(B) The yeast IPC synthase (AUR1) is down-regulated in YPH499-HIS-GAL-AUR1 cells transformed with empty vector (pRS426) incubated in non-permissive SD under the same conditions as the SMase treated samples.

NBD C_6 ceramide labelled lipid extracts fractionated by HPTLC, representative of at least three independent experiments. O, origin; IPC, inositol phosphorylceramide; SM, sphingomyelin; EPC, ethanolamine phosphorylceramide; CER, ceramide (migrating at the front). All lipid extracts normalised with respect to cell mass.



These data indicated that *Tb* SLS 4 is capable of synthesizing IPC, SM and EPC making it a novel tri-functional enzyme. In addition, it has been demonstrated to function as a 'reverse' IPC synthase, releasing ceramide from IPC at the expense of DAG (Mina *et al*., unpublished). In this it resembles the human SM synthases (Huitema *et al*., 2004). However, biochemical analyses of microsomal extracts only demonstrated IPC synthase activity, neither SM nor EPC synthase function was reconstituted (Mina *et al*., 2009). The

reasons for this remain unclear, particularly since in competition analyses the bovine liver derived donor substrates PC and PE were both shown to inhibit IPC synthase function, indicating that they bind to the enzyme.

A parallel study, using a *Leishmania* expression system, defined *Tb* SLS 4 as a dual-functional SM / EPC synthase and, importantly, confirmed by inhibition RNA (RNAi) our laboratory's observations (Mina *et al*., 2009) that the *T. brucei* enzymes are essential and so represent a new drug target for the NTD caused by these protozoa (Sutterwala *et al*., 2008). Subsequent work elsewhere used a cell-free system to analyse the function of all 4 *Tb* SLSs and defined *Tb* SLS 1 as the primary IPC synthase, *Tb* SLS 2 as an EPC synthase and *Tb* SLS 3 and 4 as the primary SM synthases (Sevova *et al*., 2010).

6.4 THE *TRYPANSOMA CRUZI* IPC SYNTHASE, *Tc* IPCS

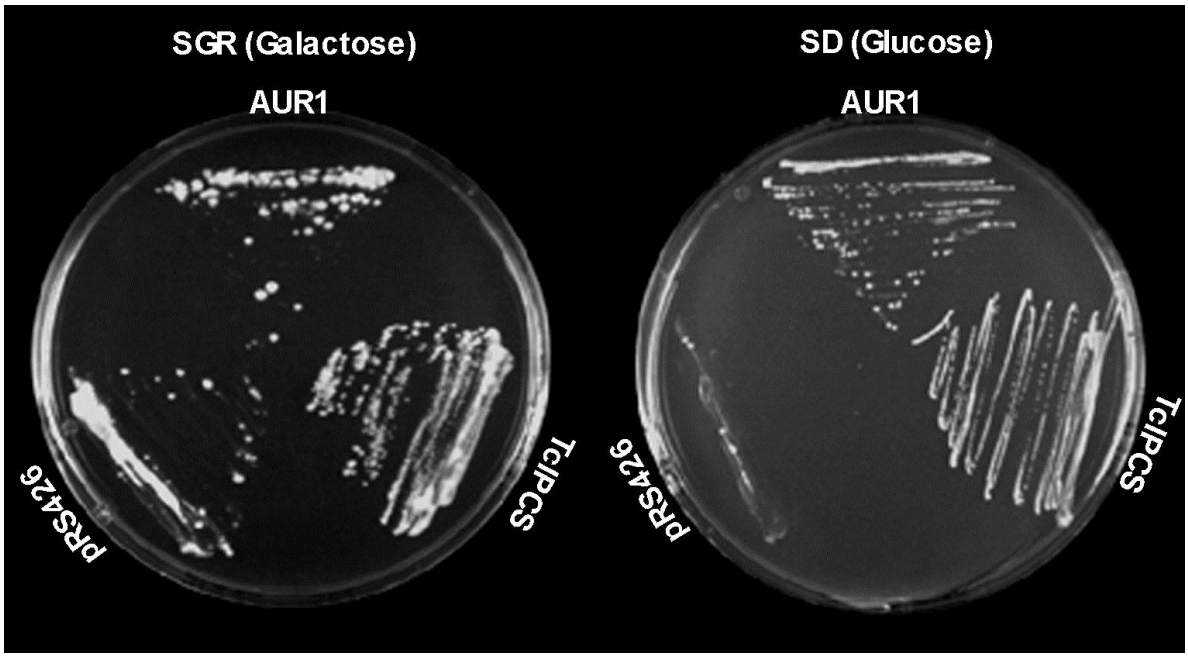
6.4.1 Identification of Tc IPCS

The insect vector carried kinetoplastid *Trypanosoma cruzi* is the causative agent of Chaga's disease (American trypanosomiasis). *T. cruzi* is known to synthesize IPC, the non-mammalian phosphosphingolipid, via a previously unidentified IPC synthase found in all stages of its lifecycle (Figueiredo *et al*., 2005). However, two very closely related (minor polymorphisms in allelic genes) orthologues of the *Tb* SLSs have now been identified in the genome database (genedb.org; *Tc* IPCS 1.1 - Tc00.1047053506885.124 and *Tc* IPCS 1.2 - *Tc*00.1047053510729.290; Denny *et al*., 2006). Again like *Lmj* IPCS, *Tb* SLS and *Tg* SLS (Chapter 5), *Tc* IPCS 1.1 and 1.2 possess the conserved D1, D2, D3 and D4 domains first identified in the SM synthase family (Denny *et al*., 2006; Sutterwala *et al*., 2008).

6.4.2 Tc IPCS is a functional orthologues of AUR1

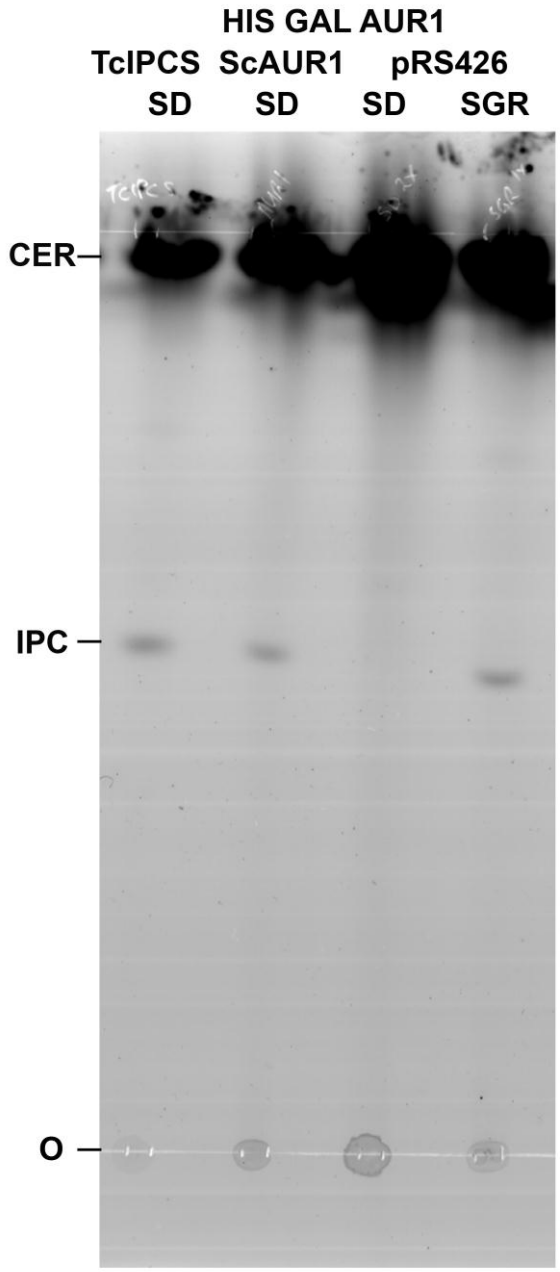
Tc IPCS open reading frames were amplified from genomic *T. cruzi* Lister 427 and ligated into the pRS426MET yeast expression vector. A single clone was chosen and sequence analysis revealed it to be *Tc* IPCS 1.2, *Tc* IPCS 1.1 was not analysed. Like the *T. brucei* orthologues *Tc* IPCS 1.2 complemented the YPH499-HIS-GAL-AUR1 mutant yeast line indicating that the *T. cruzi* enzymes are functional orthologues of AUR1p (Figure 6-6).

Figure 6-6: *Tc* IPCS 1.2 complemented the yeast AUR1 auxotrophic mutant YPH499-HIS-GAL-AUR1. Auxotrophic mutant yeast transformed with pRS426 either empty or bearing *Sc* AUR1 or *Tc* IPCS 1.2 and grown under permissive (SGR, galactose containing) or non-permissive conditions (SD, glucose containing). pRS426 - mutant yeast with empty pRS426MET25, AUR1 - mutant yeast with *Sc* AUR1; *Tc* IPCS - mutant yeast with *Tc* IPCS 1.2.



To ascertain the function of *Tc* IPCS 1.2 (and by extension *Tc* IPCS 1.1) the complemented YPH499-HIS-GAL-AUR1 mutants were metabolically labelled with NBD C_6 ceramide when grown in SD (non-permissive media; Figure 6-7).

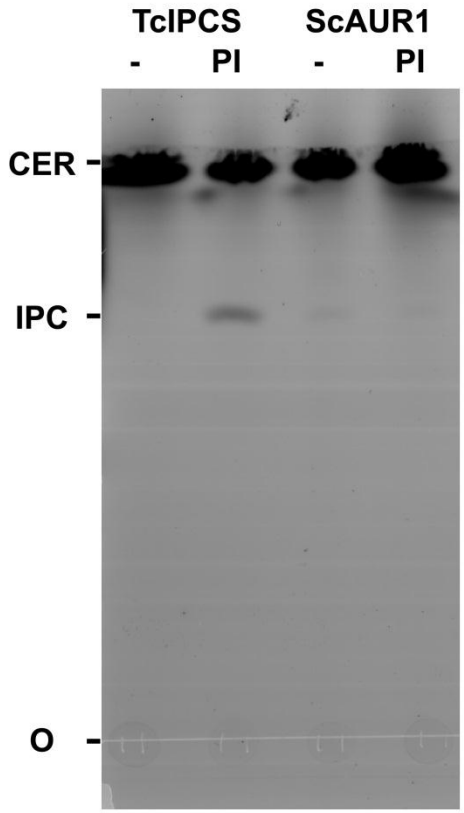
Figure 6-7: Separation by HPTLC of lipids extracted from YPH499-HIS-GAL-AUR1 yeast transformed with various plasmids following metabolic labelling using NBD C_6 ceramide in non-permissive SD (glucose as the carbon source). As expected *Sc* AUR1 complemented yeast synthesize only labelled IPC. Similarly, *Tc* IPCS 1.2 synthesized a single sphingolipid species co-migrating with IPC. The yeast IPC synthase (AUR1) is down-regulated in YPH499-HIS-GAL-AUR1 cells transformed with empty vector (pRS426) incubated in non-permissive SD, but is expressed when assayed following growth in permissive SGR (galactose as the carbon source) under the same conditions.
O, origin; IPC, inositol phosphorylceramide; CER, ceramide (migrating at the front).


HIS GAL AUR1
TcIPCS ScAUR1 pRS426
SD SD SD SGR
CER
IPC
O

6.4.3 Biochemical analyses of Tc IPCS 1.2

To confirm the functionality of *Tc* IPCS 1.2 biochemical analyses were performed as for *T. gondii Tg* SLS (Chapter 5). In brief, complemented yeast microsomes were isolated, washed with the zwitterionic detergent CHAPS to remove endogenous substrate and used in biochemical assays as described in Materials and Methodology. These microsomes, together with identically treated material from *Sc* AUR1 complemented mutant yeast, were incubated with acceptor substrate NBD C_6 ceramide and with or without donor substrate phosphatidylinositol (Figure 6-8).

Figure 6-8: HPTLC separation of lipids after incubation of isolated CHAPS-washed microsomes from *Tc* IPCS 1.2 and *Sc* AUR1 complemented YPH499-HIS-GAL-AUR1 with acceptor substrate NBD C_6 ceramide and with no donor substrate (-) or with phosphatidylinositol (PI). *Tc* IPCS 1.2 function is dependent on the addition of PI. As previously noted *Sc* AUR1 does not function with the bovine liver PI employed here (Mina *et al*., 2009). O, origin; IPC, inositol phosphorylceramide; CER, ceramide (migrating at the front).



These data demonstrate that the *T. cruzi* SLS orthologue functions as a true IPC synthases. A subsequent study elsewhere has supported this finding (*Tc* IPCS 1.1; Sevova *et al*., 2010). In this it resembles *Leishmania* species rather than the more closely related *T. brucei*. This may be hypothesized to be due to the intracellular stages of the lifecycle shared by *T. cruzi* and *Leishmania* species. In contrast, *T. brucei* is an extracellular parasite.

6.5 THE ARABIDOPSIS IPC SYNTHASE, *At* IPCS

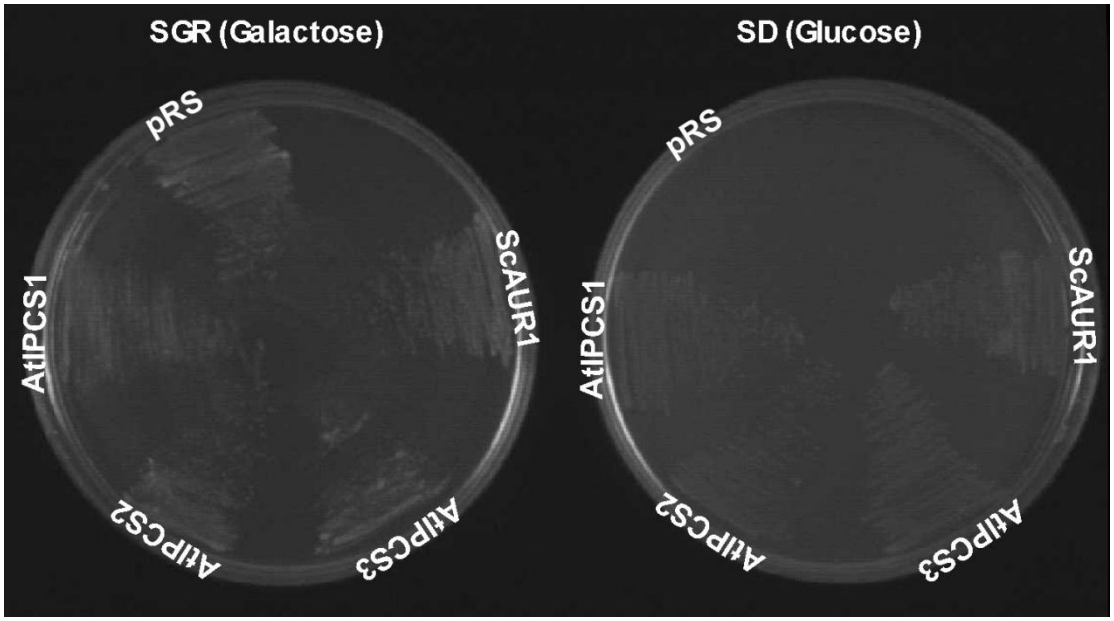
6.5.1 Identification of At IPCS 1, At IPCS 2 and At IPCS 3

Despite the presence of IPC and IPC synthase activity being long known in plant species (Bromley *et al*., 2003) the enzyme(s) responsible for synthesis remain unidentified. A homology search of the Arabidopsis genome database (Arabidopsis.org) with the protein sequence of the protozoan IPC synthase from *Leishmania major* (*Lmj* IPCS; Denny *et al*., 2006) identified open reading frames encoding three highly related sequence orthologues named as Arabidopsis IPC synthase 1 (*At* IPCS 1 – At3g54020.1), *At* IPCS 2 (At2g37940.1) and *At* IPCS 3 (At2g29525.1) demonstrating 26%, 29% and 31% identity to the protozoan protein. Subsequently, *At* IPCS 2 has been isolated, characterised and designated ERH1 (Wang *et al*., 2008).

6.5.2 At IPCS 1, At IPCS 2 and At IPCS 3 are functional orthologues of AUR1

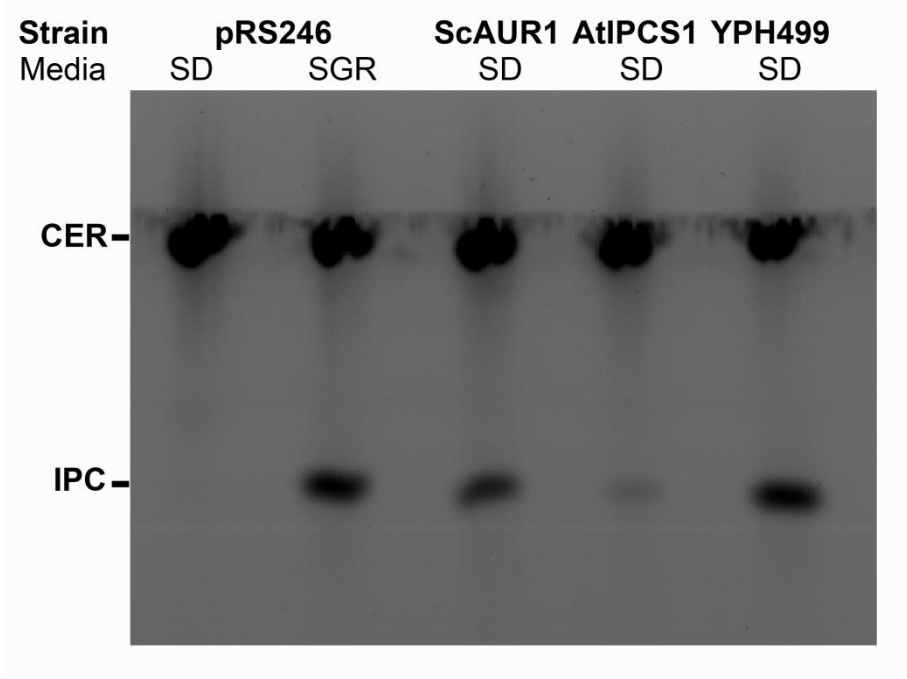
The open reading frames of *At* IPCS 1, *At* IPCS 2 and *At* IPCS 3 were cloned from cDNA into pRS426MET25 to create pRS426*At* IPCS 1-3. All three restored the growth of the AUR1 auxotrophic mutant YPH499-HIS-GAL-AUR1, as did the ectopic expression of *Sc* AUR1 (Figure 6-9). Therefore, *At* IPCS 1-3 serve as functional orthologues of the fungal AUR1p.

Figure 6-9: *At* IPCS 1-3 complemented a yeast AUR1 auxotrophic mutant YPH499-HIS-GAL-AUR1. Auxotrophic yeast transformed with pRS426 either empty or bearing *Sc* AUR1 or *At* IPCS 1-3 and grown under permissive (SGR, galactose as carbon source) or non-permissive conditions (SD, glucose as carbon source). pRS426 - mutant yeast with empty pRS426MET25, *Sc* AUR1 - mutant yeast with *Sc* AUR1; *At* IPCS 1-3 - mutant yeast with *At* IPCS 1-3.



The *At* IPCS1 complemented YPH499-HIS-GAL-AUR1 mutants were subsequently metabolically labelled with NBD C_6 ceramide when grown in SD (non-permissive media; Figure 6-10).

Figure 6-10: Separation by HPTLC of lipids extracted from YPH499-HIS-GAL-AUR1 yeast transformed with various plasmids following metabolic labelling using NBD C_6 ceramide in non-permissive SD (glucose as the carbon source). As expected *Sc* AUR1 complemented yeast synthesize only labelled IPC, as did the wild type control YPH499. Similarly, *At* IPCS 1 synthesized a single sphingolipid species co-migrating with IPC. The yeast IPC synthase (AUR1) is down-regulated in YPH499-HIS-GAL-AUR1 cells transformed with empty vector (pRS426) incubated in non-permissive SD, but is expressed when assayed following growth in permissive SGR (galactose as the carbon source) under the same conditions. IPC, inositol phosphorylceramide; CER, ceramide (migrating at the front).

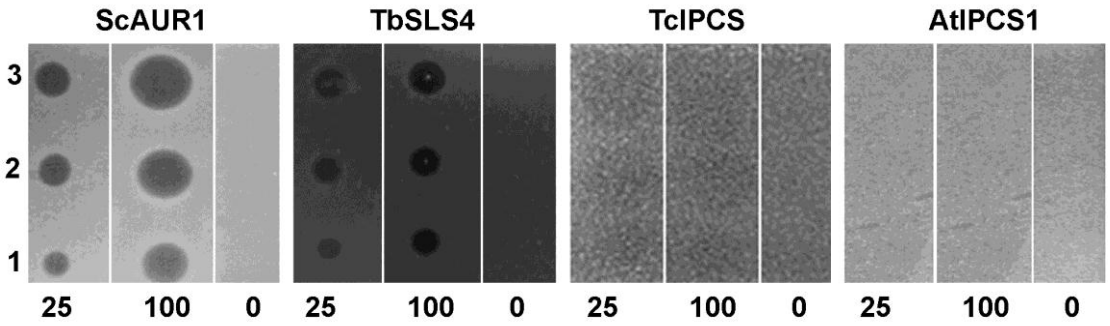


These data indicated that *At* IPCS 1 is an IPC synthase like the fungal *Sc* AUR1, although its turnover appeared low when compared to controls. These observations were subsequently confirmed by biochemical assay of *At* IPCS 1, 2 and 3 (Mina *et al*., 2010). Together with Wang *et al*., 2008 this work identified the key missing link in plant sphingolipid biosynthesis.

6.6 THE AUREOBASIDIN A SENSITIVITY OF IDENTIFIED SPHINGOLIPID SYNTHASES

Aureobasidin A (Ab A) is a specific fungal IPC synthase inhibitor (Nageic *et al*., 1997). The efficacy of Ab A was established against *Tb* SLS 4, *Tc* IPCS 1.2 and *At* IPCS 1 by diffusion assay of YPH499-HIS-GAL-AUR1 complemented yeast. *Sc* AUR1 complemented YPH499-HIS-GAL-AUR1 served as a positive control (Figure 6-11).

Figure 6-11: Agar diffusion assays of YPH499-HIS-GAL-AUR1 complemented with *Sc* AUR1, *Tb* SLS 4, *Tc* IPCS 1.2 or *At* IPCS 1. Ab A at 25 µM, 100 µM and vehicle (DMSO) were applied in 1, 2 and 3µl volumes. As expected *Sc* AUR1 was sensitive to Ab A at both concentrations. However, both *Tc* IPCS 1.2 and *At* IPCS 1, like *Tg* SLS (Chapter 5), were insensitive to Ab A. Interestingly, *Tb* SLS 4 was sensitive to Ab A at both concentrations.



Lmj IPCS complemented yeast are relatively refractory to Ab A (Denny *et al*., 2006), so perhaps the insensitivity of the *T. cruzi* enzyme to this drug is unsurprising. However, the sensitivity of *Tb* SLS 4 clearly indicates divergence, although the primary sequence data provided no clue as to the molecular mechanism of sensitivity / resistance. Although, notably *T. brucei* bloodstream form parasites were also sensitive to Ab A (Mina *et al*., 2009; Sevova *et al*., 2010). In addition, microsomal assay indicated that the IC_{50} of *Tb* SLS 4 is < 1 nM (Mina *et al*., 2009). Extracts from wax beans showed IPC synthase to be

similarly Ab A sensitive, < 1 nM (Bromley *et al*., 2003). In contrast *At* IPCS 1-3 has been shown by diffusion (Figure 6-11) and biochemical assay (Mina *et al*., 2010) to be Ab A resistant. This indicated that subtle features of these evolutionarily divergent IPC synthases define Ab A sensitivity / resistance.

SUMMARY

These data clearly demonstrate that SM synthesis alone (from *Hs* SMS 1 or 2) is not able to compensate for the lack of IPC synthase activity in the auxotrophic AUR1 mutant, YPH499-HIS-GAL-AUR1 (Figure 6-2). This indicated that the IPC synthase function of *Tg* SLS that complements for the AUR1 defect (Chapter 5).

Like *T. gondii* the kinetoplastid parasite *Trypanosoma brucei* (the causative agent of African sleeping sickness, African trypanosomiasis) has been shown to possess both SM (Patnaik *et al*., 1993) and IPC (Guther *et al*., 2006). The identification of orthologues of the characterised *Leishmania* IPC synthase (Denny *et al*., 2006) allowed analyses of the *T. brucei* enzymes. Both *Tb* SLS 1 and *Tb* SLS 4 complement the YPH499-HIS-GAL-AUR1 mutant indicating that they possess a functional IPC synthase (Figure 6-3). However, like *Tg* SLS, both produce products co-migrating with IPC and SM markers, *Tb* SLS 4 also synthesizes a sphingolipid co-migrating with EPC (Figure 6-4). Analysis confirmed that the product from *Tb* SLS 4 was SM (Figure 6-5), but *in vitro* studies demonstrated only IPC synthase activity (Mina *et al*., 2009). In this *Tb* SLS 4 resembled *Tg* SLS and the SM synthase action of both remains unclear, although analyses elsewhere have indicated that *Tb* SLS 4 is the principle SM synthase in *T. brucei* and *Tb* SLS 1 the primary IPC synthase (Sevova *et al*., 2010).

Notably unlike the closely related *T. brucei* enzymes, the *Trypanosma cruzi* (Chaga's disease, American trypanosomiasis) orthologue functioned only as an IPC synthase, as demonstrated by metabolic labelling (Figure 6-7) and biochemical analysis (Figure 6-8). Is this it resembled the related *Leishmania* (Denny *et al*., 2006) and distant Arabidopsis (Figure 6-10; Mina *et al*., 2010) enzymes? No indication of the basis for the functional diversity of the *T. brucei* enzyme compared to closely related kinetoplastid orthologues is

apparent from the primary sequence data. However, this diversity was further evidenced by the sensitivity of *Tb* SLS 4 to aureobasidin A (Ab A), a potent inhibitor of the fungal enzyme (Figure 6-11; Mina *et al*., 2009). In contrast, the *Leishmania* (Denny *et al*., 2006) and *T. cruzi* (Figure 6-11) enzymes are resistant to Ab A. This result was surprising, however it should be noted that whilst the Arabidopsis IPC synthase is resistant to the drug (Figure 6-11; Mina *et al*., 2010) the enzyme from wax beans is, like *Tb* SLS 4, acutely sensitive (Bromley *et al*., 2003).

Therefore the molecular basis of Ab A sensitivity / resistance and IPC / SM / EPC synthase function remains unknown. However, the functional characterisation of orthologues from mammals, plants, protozoa and fungi should facilitate further detailed studies in the future.

CHAPTER 07

THE EVOLUTION OF THE EUKARYOTIC SPHINGOLIPID SYNTHASE

7 THE EVOLUTION OF THE EUKARYOTIC SPHINGOLIPID SYNTHASE

As discussed above the identified *T. gondii* and *P. falciparum* sphingolipid synthases share conserved domains (1-4) with the previously identified animal SMS's (Huitema *et al*., 2004) and kinetoplastid protozoan SLS's (Denny *et al*., 2006; Sutterwala *et al*., 2008). Domains 3 and 4 contain the residues forming the catalytic triad of the enzyme and these, but not domains 1 and 2, are conserved in both fungi and plants (Mina *et al*., 2010 a). This lack of domains 1 and 2 has been hypothesized to be related to the use of ceramide by the animal and kinetoplastid enzymes compared with phytoceramide by plants and fungi (Mina *et al*., 2010 a). However, there is no evidence for this.

The catalytic triad believed to form from domains 3 and 4 promotes nucleophilic attack on lipid phosphate ester bonds, this is thought to lead to the transfer of choline- or inositol phosphate from phosphoglycero lipids to ceramide or phytoceramide to form SM and IPC respectively. The conservation of the catalytic triad in the eukaryotic SLSs indicates that they share a common mechanism of action and are likely to have evolved from a common ancestor. Conversely, the prokaryotic SMS lacks an identifiable catalytic triad and is likely to have evolved independently (Luberto *et al*., 2003). Previous phylogenetic analyses have shown that the plant, kinetoplastid, animal and plant SLS's form distinct but related clades. Given the functional identification of the apicomplexan SLS in this study it was decided to repeat the analysis with the addition of the *T. gondii* and *P. falciparum* sequences.

7.1 PHYLOGENETIC ANALYSES OF THE EUKARYOTIC SPHINGOLIPID SYNTHASES

7.1.1 Sequence alignment

Predicted protein sequences from representative animal, plant, fungal and protozoan species were selected from the genome databases. Following an initial Clustal W alignment of the complete protein sequences a region of relative conservation was chosen for further analyses, this represented amino acids 92 to 362 of the identified *T. gondii* enzyme. This region contained the conserved domains 1-4 of the animal SMS's. Realigning the conserved region using Clustal W gave the alignment shown in Figure 7-1.

Figure 7-1: Sequence alignment of a region of the eukaryotic SLS corresponding to amino acids 92-362 of the *T. gondii* enzyme (*Tg* SLS). In the mammalian SMS's this region contains the conserved domains 1-4. **Domain 1**, **domain 2**, **domain 3** and **domain 4** are illustrated in *Hs* SMS 1 and in the apicomplexan sequences. The 2 histidine (**H**) and aspartate (**D**) residues forming the catalytic triad are highlighted in bold in all proteins. The human lipophosphate phosphatase (*Hs* LPP 1), a hydrolase, is predicted to possess the same mode of action with respect to a catalytic triad (Sigal *et al*., 2005). In these analyses *Hs* LPP 1 functions as an out group, i.e. a protein that is related but of a different family that allows the consideration of the evolution of SLS evolution.

Homo sapiens LPP 1 (out group) accession number: O14494; *T. gondii* SLS: 50.m03113; *P. falciparum* SMS 1&2: PFF1210w and PFF1215w; *Arabidopsis thaliana* IPCS 1-3: At3g54020.1, At2g37940.1, At2g29525.1; *T. brucei* SLS 1-4: Tb09.211.1030, Tb09.211.1020, Tb09.211.1010, Tb09.211.1000; *T. cruzi* IPCS 1&2: Tc00.1047053506885.124, Tc00.1047053510729.290; *L. major* IPCS: LmjF35.4990; *Aspergillus fumigatus* AUR1p: AAD22750; *Candida albicans* AUR1p: AAB67233;

Pneumocystis carinii AUR1p: CAH17867; *Saccharomyces cerevisiae* AUR1p: NP_012922; *Schizosaccharomyces pombe* AUR1p: Q10142; *Caenorhabditis elegans* SMS1-3: Q9U3D4, AAA82341, AAK84597; *Homo sapiens sapiens* SMS 1&2: AB154421, Q8NHU3; *Mus musculus* SMS 1&2: Q8VCQ6, Q9D4B1.

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TbSLS2      ERMPDPKVTK PLPDLGFELL TKVPGMYVLA DCCIGFLNIL SVFTAFKLYL
TbIPC4      ERMPDPKVTK PLPDLGFELL TKVPGMYVLA DCCIGFLNIL SVFTAFKLYL
TbIPC3      ERMPDPKVTK PLPDLGFELL TKISFLSVVT DVLIAFLSSL SFFTLWKLYL
TbIPC1      --MPDPKVTK PLPDLGFEVL HKYPFLFSVA DCCIGFLNIL SVFTAFKLYL
TcIPC1      ARMPDPKVVR PLPDIGFEVF PKVGWLEHVT DVCIFILNFL SLLVAFKLYL
TcIPC2      ARMPDPKVVR PLPDIGFEVF PKVGWLEHLT DVCIFILNFL SLLVVFKLYL
LmIPCS      ARMPDPEKVR PLPDLLLESI PKVALLENGT NVIIFLLNAT TVVVGFKVFL
HsSMS1      GMPNGYRK-- EMIKIPMPEL ERSQYPMEWG KTFLAFLYAL SCFVLTTVMI
MmSMS1      GMPNGFRK-- EMIKIPMPEP ERSQYPMEWG KTFLAFLYAL SCFVLTTVMI
HsSMS2      GLRKGTKKYP DYIQIAMPTE SRNKFPLEWW KTGIAFIYAV FNLVLTTVMI
MmSMS2      GLRKGAKKYP DYIQISMPND SKNKFPLEWW KTGIAFVYAL FNLILTTVMI
AtIPC1      EITTEIGLLA ENWKYLLAGL LCQYIHGLAA RGVHYIHRPG PTLQDSGFFV
AtIPC3      EITTEIGRLA ENWKYLLAGI LCQDIHGLAA KGGHDIHRPG TTLQDSGFFV
AtIPC2      EISTEIGLLA ENWKYLLAGL ICQYIHGLAA KGVHYIHRPG PTLQDLGFFL
AfAUR1      FTLLLVSLLL PITRQFFLPF LPIAGWLIFF YACQFIPSDW RPAIWVRVLP
SpAUR1      ACLLAIALTV PLTRQIFFPA IVIITWAILF YSCRFIPERW RPPIWVRVLP
PcAUR1      VLLYVLLLIF PITRMFFRPF LPIVSWLILF YACRFIPSSM RPHIWISILP
CaAUR1      ILAFATCFLI PLTSQFFLPA LPVFTWLALY FTCAKIPQEW KPAITVKVLP
ScAUR1      YCFLGTLFII PATSQFFFNA LPILTWVALY FTSSYFPDDR RPPITVKVLP
CeSMS1      PLLPDEGPSH AVRLEMPGDK PASPHDRFPK TPLKTLVAFL MLVVAAAGNT
CeSMS2      INIDPIDPEP TPIRKEFTCE DTFHHEHHGN SEGFKTLTAF LCLMLSAFLN
CeSMS3      MGSVSKTVIS ARGASPDDEQ NGTKNGISNG SEWAKCIFLF FFLFIAGMSN
PfSMS1      YFEGQENIKK SVEIKNCPYK KKMLKIFLRR LVCATIIGII GICIQCYFII
PfSMS2      MSNISISRNS TINEEEILSE YRLCKILLIK LMFALLFLLI ALIIQGFFMI
TgSLS       VVDAVTVGII IVTIFRHAAF LKVPLNLLVG TRFLFLLGSL YMCRGIAIII
HsLPP1      SRHTPFQRGV FCNDESIKYP YKEDTIPYAL LGGIIIPFSI IVIILGETLS

TbSLS2      LHRHCVGSGE PELPCNIPGV SRFFLSVWLC KENCR----- IELRNVHTIA
TbSLS4      LHRHCVGSGE PELPCNIPGV SRFFLSVWLC KENCR----- IELRNVHTIA
TbSLS3      LHRHCVGSGE PELPCNIPGV SRFFLSVWLC KENCR----- IELRNVHTIA
TbSLS1      LHRHCVGSGE PELPCNIPGV SRFFLSVWLC KENCR----- IELRNVHTIA
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TcIPC1      LHRQNEGLDE LQPFSCCPLI GKIIFGVWDR GRQSG----- IEKRDAHLIV
TcIPC2      LHRQNEGLDE LQPFSCCPLI GKIIFGVWDS GRQSG----- IEKRDAHLIA
LmjIPC      LERHMNGLPR VTFLVGVPKI GSFLNRMAFG VLDSGRRP-- FPLKNVFPIM
HsSMS1      SVVHERVPPK EVQPPLPDTF FDHFNRVQWA FSICEING-- MILVGLWLIQ
MmSMS1      SVVHERVPPK EVQPPLPDTF FDHFNRVQWA FSICEING-- MILVGLWLFQ
HsSMS2      TVVHERVPPK ELSPPLPDKF FDYIDRVKWA FSVSEING-- IILVGLWITQ
MmSMS2      TVVHERVPPK ELSPPLPDKF FDYFDRVKWA FSVSEING-- MVLVGLWITQ
AtIPC1      LPELGQDKGF ISETVFTCVF LSFFLWTFHP FIVKS----- --KKIYTVLI
AtIPC3      LPELGQHKGF ISETAVTCGF PSVFLGTSQP FIVKS----- --KKIYTVLI
AtIPC2      LPELGQERSY ISETVFTSVF LSFFLWTFHP FILKT----- --KKIYTVLI
AfAUR1      ALENILYGAN ISNILSAHQN VVLDVLAWLP YGICHYGA-- PFVCSAIMFI
SpAUR1      TLENILYGSN LSSLLSKTTH SILDILAWVP YGVMHYSA-- PFIISFILFI
PcAUR1      TLENILYGVR LSSLLSNHTH FFLDILAWIP YGIIHFGA-- PFITAIVIYL
CaAUR1      AMETILYGDN LSNVLATITT GVLDILAWLP YGIIHFSF-- PFVLAAIIFL
ScAUR1      AVETILYGDN LSDILATSTN SFLDILAWLP YGLFHFGA-- PFVVAAILFV
CeSMS1      ITLSWIHERY PLTPPLPDIV FELIPKIPWG LRLCENLM-- IGSFVSLLVL
CeSMS2      FFLLTVIHDV VPRQPLPDLT FMIIPQQRWA WSVGDVLS-- TVSSVVAFTI
CeSMS3      WAVLAYTHDY VPRESLPDIV FSLVSEQRWA SSLGDFCV-- ALCIVMLGAL
PfSMS1      LSDTYYKTGD EPLKDRLHEI FKEIPAFMNT PFVNGSIMFF LAITLLRFGL
PfSMS2      YSDSYYKSNT QPLSDRIHDL FG-NPPKWIS YKLSNTLIAI LTLSFLKIIL
TgSLS       TTVPPSRRNC VPPPVMSVGS FFYLGVLQMF SMRNECTGMI ISGHSTITCC
HsLPP1      VYCNLLHSNS FIRNNYIATI YKAIGTFLFG AAASQSLT-- ----------


TbSLS2      WI-------- --RFITSYAL LLLFRSVVIV MTSLPAPDDL CQ--DPPKIE
TbSLS4      WI-------- --RFITSYAL LLLFRSVVIV MTSFPAPDDL CQ--NPPKIE
TbSLS3      WI-------- --RFITSYAL LLLFRSLVIV MTSMPTPVDK CQ--NPPKIE
TbSLS1      WI-------- --RFITSYAL LLLSRSVIMV VTSLPNPDDL CQ--DPPKIE
TcIPC1      WI-------- --RYFTTYFI VMLFRAIVVV MTSYPATDNH CQ--NPVKIT
TcIPC2      WI-------- --RYFTTYFI VLLFRAIVVV MTSYPATDNH CQ--NPMKIT
LmjIPC      AI-------- --RFLTSYAV VMVFRAFVIM GTSYPATDNH CQ--NPQVIE
HsSMS1      WLLLKYKSII SRRFFCIVGT LYLYRCITMY VTTLPVPGMH FN--CSPKLF
MmSMS1      WLLLKYKSII SRRFFCIVGT LYLYRCITMY VTTLPVPGMH FN--CSPKLF
HsSMS2      WLFLRYKSIV GRRFCFIIGT LYLYRCITMY VTTLPVPGMH FQ--CAPKLN
MmSMS2      WLFLRYKSIV GRRFFFIMGT LYLYRCITMY VTTLPVPGMH FQ--CAPKLN
AtIPC1      WC-------- --RVLAFLVA CQFLRVITFY STQLPGPNYH CR--EGSELA
AtIPC3      WC-------- --RVLAFLVA CQFLRVITFY STQLPGPNYH CR--EGSELA
AtIPC2      WC-------- --RVLAFLVA CQFLRVITFY STQLPGPNYH CR--EGSKVS
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AfAUR1     FGPPGTVPLF ARTFGYISMA AVTIQLFFPC SPPWYENLYG LA--PADYSM
SpAUR1     FAPPGTLPVW ARTFGYMNLF GVLIQMAFPC SPPWYENMYG LE--PATYAV
PcAUR1     SSPPGTLPVF AKCFGYLNLI GVIIQLVFPC SPPWYESLYG SQ--LANYSM
CaAUR1     FGPPTALRSF GFAFGYMNLL GVLIQMAFPA APPWYKNLHG LE--PANYSM
ScAUR1     FGPPTVLQGY AFAFGYMNLF GVIMQNVFPA APPWYKILYG LQ--SANYDM
CeSMS1     ILFHRHRWIV LRRLCFIGSI LYGMRCITMM VTPVPKADED FE--CSPRFG
CeSMS2     IFLHHQRWIV LRRTFLLGAI MYGLRAVILG VTFLPPSFHN RDEICQPQVN
CeSMS3     LVIHQHRGTI LKRVVFCAGT LYAMRSVTLA ATQLPSGYTD NQGRCRDQVE
PfSMS1     FCPLLLSITI LIRIILMLSF IYCIRSFFIY VTTLPCPIPT CQPLKHKTLV
PfSMS2     FNSIYLSIAI ICRFLYIVGS FYIIRGLLIY VTSLPATLET CLPLESGNFL
TgSLS      CMATWLLYGN ANREDQDTKG VLFYEVARFL ATRLQKVRAS ARQLTTQYAI
HsLPP1     ---------- ----DIAKYS IGRLRPHFLD VCDPDWSKIN CSDGYIEYYI

TbSLS2     NPVKNVILTV ---------- --LTAGGGSI HCGDLMYSGH TVILTLHLMF
TbSLS4     NPVKNVILTV ---------- --LTAGGGSI HCGDLMYSGH TVILTLHLMF
TbSLS3     NPVKNVILTV ---------- --LTAGGGSI HCGDLMYSGH TVILTLHLMF
TbSLS1     NRVKDVILTV ---------- --LTAGAGSI HCGDLMYSGH TVILTLHLMF
TcIPC1     NPVKNVIMTL ---------- --VTFGSGFI HCGDLMFSGH TVPITLSLLV
TcIPC2     NPVKNVIMTL ---------- --VTFGSGSI HCGDLMFSGH TVSITLSLLV
LmjIPC     HPVLNVILTL ---------- --VTLGSGAI HCGDLMFSGH TMILSLAFIL
HsSMS1     GDWEAQLRRI MKLIAGGG-- --LSITGSHN MCGDYLYSGH TVMLTLTYLF
MmSMS1     GDWEAQVRRI MKLIAGGG-- --LSITGSHN MCGDYLYSGH TVMLTLTYLF
HsSMS2     GDSQAKVQRI LRLISGGG-- --LSITGSHI LCGDFLFSGH TVTLTLTYLF
MmSMS2     GDSQAKIQRI LRLISGGG-- --LSITGSHI LCGDFLFSGH TVVLTLTYLF
AtIPC1     RLPRPHNVLE VLLLNF---- ----PRGVIY GCGDLIFSSH MIFTLVFVRT
AtIPC3     RLPRPHNVLE VLLLNF---- ----PRGVIY GCGDLIFSSH MIFTLVFVRT
AtIPC2     RLPWPKSALE VLEIN----- ----PHGVMY GCGDLIFSSH MIFTLVFVRT
AfAUR1     PGNPAGLARI DELFG----- --IDLYTSGF RQSPVVFGAF PSLHAADSTL
SpAUR1     RGSPGGLARI DALFG----- --TSIYTDGF SNSPVVFGAF PSLHAGWAML
PcAUR1     GGSAGGLARI DSFFG----- --TKVYTSIF PASPLVFGAF PSLHSGHATL
CaAUR1     HGSPGGLGRI DKLLG----- --VDMYTTGF SNSSIIFGAF PSLHSGCCIM
ScAUR1     HGSPGGLARI DKLLG----- --INMYTTAF SNSSVIFGAF PSLHSGCATM
CeSMS1     ENATFSLIVM RGVWSMFGLG LNLFDNQKVV LCGDYIYSGH TLVLVVSALF
CeSMS2     RTAMYGMEIA TRFLTYVIT- LGLTSGQDKI LCGDLMFSGH TVVLTIMYFV
CeSMS3     SEAGVFFGRL FEQTIRIG-- ---FQSKDQM LCGDLLFSGH TLVMVTCSLA
PfSMS1     ENLYTFYLII ---------- -----TAQVY ECTDLVISGH TAFTTLLTFF
PfSMS2     FNLLQIIKIN ---------- -----TNLVY VCADLIVSGH SFSTTIFLMF
TgSLS      TPAFDVEEGS ---------- -----ETVPQ AESEEELSSA SGVFVQEDRR
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HsLPP1      CRGNAERVKE ---------- -----GRLSF YSGHSSFSMY CMLFVALYLQ


TbSLS2      HWIYGAMVH- --WSFRPVVT VVAIFGYYCI VASRFHYTDD VLVAIYLTIA
TbSLS4      HWIYGAMVH- --WSFRPVVT VVAIFSYYCI VASRFHYTDD VLVAIYLTIA
TbSLS3      HWIYGAMVH- --WSFRPVVT VVAIFGYYCI VASRSHYTDD VLVAIYLTIA
TbSLS1      HWIYGAMVH- --WSFRPVVT VVAIFGYYCI VASRFHYTDD VLVAIYLTIA
TcIPC1      QWIYGSMLH- --WVFRPASV LLVLLSFYSI IASRSHYTDD ILVSFYITVT
TcIPC2      QWIYGSMLH- --WVFRPASV LLVLLSFYSI IASRSHYTDD ILVSFYITVT
LmjIPC      AWDYSPFLHP --WAVRVWVS VLLPISYYCI LASRSHYTDD ILVAMYVMIA
HsSMS1      IKEYSPRRL- --WWYHWICW LLSVVGIFCI LLAHDHYTVD VVVAYYITTR
MmSMS1      IKEYSPRRL- --WWYHWICW LLSVVGIFCI LLAHDHYTVD VVVAYYITTR
HsSMS2      IKEYSPRHF- --WWYHLICW LLSAAGIICI LVAHEHYTID VIIAYYITTR
MmSMS2      IKEYSPRHF- --WWYHLVCW LLSAAGIICI LVAHEHYTVD VIIAYYITTR
AtIPC1      YQKYGSKRF- ----IKLLGW VIAILQSLLI IASRKHYTVD VVVAWYTVNL
AtIPC3      YQKYGSKRF- ----IKLLGW VIAILQSLLI IASRKHYTVD VVVAWYTVNL
AtIPC2      YQKYGTKRF- ----IKLFGW LTAIVQSLLI IASRKHYSVD VVVAWYTVNL
AfAUR1      AALFMSQVF- --PRLKPLFV IYTLWMWWAT MYLSHHYAVD LVGGGLLATV
SpAUR1      EALFLSHVF- --PRYRFCFY GYVLWLCWCT MYLTHHYFVD LVGGMCLAII
PcAUR1      EALFLSCIF- --PKTTPYFV IYVLWLWWCT MYLTHHYFID LIGGSCLAVF
CaAUR1      EVLFLCWLF- --PRFKFVWV TYASWLWWST MYLTHHYFVD LIGGAMLSLT
ScAUR1      EALFFCYCF- --PKLKPLFI AYVCWLWWST MYLTHHYFVD LMAGSVLSYV
CeSMS1      IGEYSPRRF- --YILHWLSW LVCSVGVIFL VLSHGHYTID VILSYFACTR
CeSMS2      QLQYTPRGL- --VILRYIAA PITFLGIAAL VVSGGHYTMD VLIAYWLTSH
CeSMS3      VAYYLPKSI- --KPLQWVSH VACLIGMICM TISRTHYTID VVIAYWLSNM
PfSMS1      WFFYERNIY- ----VKTTIF LYSIYIYIII IISRFHYTVD VLMGYVFGGS
PfSMS2      SFYYINNVI- ----IKFIIF TFSCFIYAII IIGFIHYTSD VLLGIIFGVF
TgSLS       KTRVAAFLRL --NLLRSLCL VVGVVNLCLI VCSFNHYSID VFMALNFTFG
HsLPP1      ARMKGDWARL LRPTLQFGLV AVSIYVGLSR VSDYKHHWSD VLTGLIQGAL


TbSLS2      TFIAVGHNAD GAPWQLQLFI RWLPCCGANS REMTEDSQ
TbSLS4      TFIAVGHNAD GAPWQLQLFI RWWPCCGANS REMTEDSQ
TbSLS3      TFIAVGHNAD GAPWQLQLFI RWLPCCGANS REVTEDSQ
TbSLS1      TFIAVGHNAD GAPWQLQLFI RWLPCCGANS REVTEDGV
TcIPC1      TFLVLRHSPE GAPWQLQLLI GWWPCCASNE EAEDSDRH
TcIPC2      TFLVLRHSPD GAPWQLQLLI GWWPCCVSNE ETEDSDRN
LmjIPC      TYKVIDHAET GAPWQMQLLI RWMPWPGANT IEKWTADE
HsSMS1      LFWWYHTMAN QQVLKEASQM NLLARVWWYR PFQYFEKN
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MmSMS1      LFWWYHTMAN QQVLKEASQM NLLARVWWYR PFQYFEKN
HsSMS2      LFWWYHSMAN EKNLKVSSQT NFLSRAWWFP IFYFFEKN
MmSMS2      LFWWYHSMAN EKNLKVSSQT NFLSRAWWFP IFYFFEKN
AtIPC1      VVFFLDKKLP ELPDRTTALL PVISKDRTKE ESHKLLNG
AtIPC3      VVFFLDKKLP ELPDRTTALL PVISKDRTKE ESHKLLNG
AtIPC2      VVFCLDKKLP ELPDRTAVLL PVISKDRTKE ENHKLLNG
AfAUR1      AFYFAKTRFM PRVQNDKMFR WDYDYVE-YG DSALDYGY
SpAUR1      CFVFAQKLRL PQLQTGKILR WEYEFVI-HG HGLSEKTS
PcAUR1      IFYIANYNYS SHIYSRNLFR WDYHNVI-YD SSKTDPEV
CaAUR1      VFEFTKYKYL PKNKEGLFCR WSYTEIEKID IQEIDPLS
ScAUR1      IFQYTKYTHL PIVDTSLFCR WSYTSIEKYD ISKSDPLA
CeSMS1      VFWAYHTQAA HPSIRLSVQN HQAKEFWFPL LRWFEGDI
CeSMS2      VFWSYHQIFE MRKDDRPQAP LSRLWWFWLC YWFESDVA
CeSMS3      VFRMYHAYCE VDMCMERRKS ILYSWWPCRI VDWLEQDI
PfSMS1      VFLFYHYLVD VAARRYALNT SVFPQTYGFS GSFTDRFQ
PfSMS2      MFSFYHIMLD ISSQYYIFNK LFEIKIISNN KNIHAKPF
TgSLS       AWCLYHCILS LIWAEKEERD KRQKVALLRS EGIAAGAA
HsLPP1      VAILVAVYVS DFFKERTSFK ERKEEDSHTT LHETPTTG
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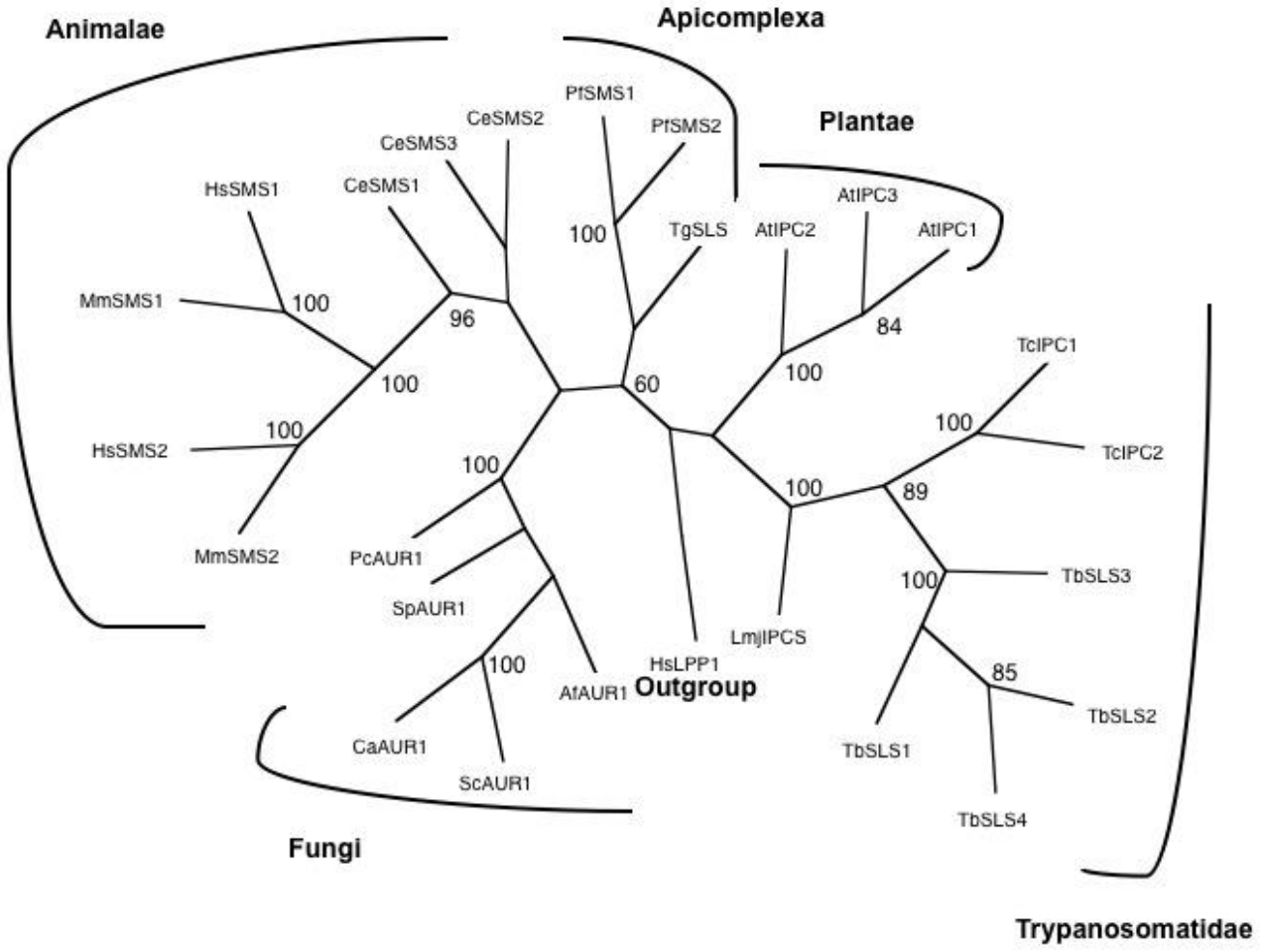
It can be seen that the *Tg* SLS domain 1 does not align with the equivalent domains from either the apicomplexan *P. falciparum* enzyme or that from mammals, in fact it is not even present in the region analyzed. Similarly, whilst *Pf* SMS 1 and 2 domains 2 and 3 clearly align with *Hs* SMS 1, these regions of *Tg* SLS do not. In this *Tg* SLS resembles the fungal sequences used in this analysis with respect to domain 3. However, domain 4 is perfectly aligned in all sequences analyzed (Figure 7-1). This indicates that the *T. gondii* enzyme is divergent with respect to its primary sequence (only 27-28% identity to *Pf* SMS; chapter 5). However, it is clearly functional and topology prediction indicated that despite possessing 8, rather than the more common 6, transmembrane domains the catalytic triad can still form.

7.2 PHYLOGENETIC ANALYSES OF THE NEWLY EXPANDED EUKARYOTIC SPINGOLIPID SYNTHASE (SLS) FAMILY

To begin to understand the evolutionary relationships of the newly expanded eukaryotic SLS family a phylogenetic analyses were undertaken using the alignment shown in Figure 7-1. Using a Maximum Parsimony algorithm as described in Materials and Methodology, a single tree, shown in Figure 7-2, was generated (http://evolution.genetics.washington.edu/phylip.html). Under Maximum Parsimony the simplest model of evolution is used where the most direct path of the mutation of one residue to another is assumed. As discussed above *Tg* SLS did not fully align with the other SLS proteins analysed, however in this preliminary analysis the complete data set (including gaps) was used to avoid discarding potentially useful information. Subsequent studies may consider focusing on the region including only D4 which aligned well across all sequences chosen (Figure 7-1).

Figure 7-2: Maximum parsimony analyses of Animalae, Fungi, Trypanosomatidae, Plantae and Apicomplexa sphingolipid synthase sequences. Bootstrap scores >60 shown, this indicates the number of times out of 100 analyses this node is reproduced and thus is a measure of certainty. Accession numbers as in Figure 7-1.


Animalae
Apicomplexa
Plantae
Fungi
Trypanosomatidae
Outgroup
PfSMS1
PfSMS2
CeSMS2
CeSMS3
CeSMS1
HsSMS1
MmSMS1
HsSMS2
MmSMS2
TgSLS
AtIPC3
AtIPC2
AtIPC1
TcIPC1
TcIPC2
TbSLS3
TbSLS2
TbSLS1
TbSLS4
LmjIPCS
HsLPP1
PcAUR1
SpAUR1
AfAUR1
CaAUR1
ScAUR1
100
96
100
100
100
60
100
84
100
100
100
89
100
85
100

Despite the divergence of *Tg* SLS from *Pf* SMS, the apicomplexan enzymes clearly form a new clade of SLS proteins. This study therefore expands the repertoire of known SLS's to the Apicomplexa and reinforces the hypothesis that these key enzymes share a conserved mechanism of action and are derived from a common eukaryotic ancestor. Furthermore, in relation to the out group (*Hs* LPP 1) the apicomplexan sequences group with the Animalae and Fungi. In contrast, the Trypanosomatidae group with the Plantae (Figure 7-2).

CHAPTER 08

DISCUSSION

8 DISCUSSION

Sphingolipids are ubiquitous in the Eukaryota and sphingolipid biosynthetic pathway is largely conserved across eukaryotic evolution. Complex sphingolipids and their precursors are involved in many cellular processes including cell signalling (e.g. for apoptosis via ceramide) and the formation of functional lipid microdomains, rafts (Futerman and Hannun, 2004). However, there is a major divergence after the formation of ceramide species, in mammals, the major complex sphingolipid is sphingomyelin (SM) whereas in kinetoplastid protozoans, fungi and plants inositol phosphoryl ceramide (IPC) is the primary phosphosphingolipid (Dickson and Lester, 1993; Sperling and Heinz, 2003; Denny *et al*., 2006).

IPC is not found in mammalian systems and enzyme responsible for its synthesis, IPC synthase (AUR1 in yeast), has been identified as a target for novel antifungal agents (Nagiec *et al*., 1997). In support of this several specific inhibitors have been demonstrated to have potent antifungal activity (Georgopapadakou, 2000) which is believed to occur through the accumulation of the acceptor substrate phytoceramide which induces apoptosis (Fischl *et al*., 1986). The enzyme responsible for mammalian SM synthesis has also been characterized (Huitema *et al*., 2004). It employs the same mode of action as AUR1p, utilizing a catalytic triad with an activated histidine residue in the active site (Huitema *et al*., 2004), and is essential for mammalian cell growth (Tafesse *et al*., 2007).

More recently, the IPC synthase of the kinetoplastid protozoan parasite *Leishmania major* (*Lmj* IPCS) was isolated and characterized (Denny *et al*., 2006). It is encoded by a single copy gene and its mode of action has been subsequently explored and found to resemble that of its sister enzymes in yeast and mammals (Mina *et al*., 2010 b and 2011).

Orthologues of *Lmj* IPCS were found in the related kinetoplastids *Trypanosoma brucei* and *T. cruzi*, but these remained uncharacterized until the start of this study (Denny *et al*., 2006).

In contrast, the gene encoding plant IPC synthase remained unidentified before the work reported here, although the enzyme activity has been recorded in bean microsomes (Bromley *et al*., 2003). Similarly, the enzymes responsible for phosphosphingolipid biosynthesis (SM, IPC or ethanolamine phosphorylceramide: EPC) in the apicomplexan protozoan parasites remain little studied. Although, *Plasmodium falciparum* (the causative agent of lethal cerebral malaria) encodes 2 orthologues (*Pf* SMS 1 and 2) of the mammalian enzymes (Huitema *et al*., 2004) and is known to synthesize SM (Haldar *et al*., 1991; Gerold and Schwarz, 2001). The related, model apicomplexan *T. gondii* (Kim and Weiss, 2008) has been shown to harbour SM and, compared to the host cell, large quantities of EPC (Welti *et al*., 2007). In addition, metabolic labelling studies indicated *de novo* synthesis of SM (Azzouz *et al*., 2002) and IPC (Sonda *et al*., 2005), however the enzyme(s) responsible for these activities remained unknown.

The parasitic lifestyle means that a pathogen may be able to scavenge enough sphingolipid to make synthesis non-essential or even redundant. For example, *T. gondii* can both scavenge and synthesize phosphatidylcholine (PC) *de novo* (Charron and Sibley, 2002). The balance between these two sources is unclear, but when host PC is restricted *de novo* synthesis is increased (Charron and Sibley, 2002). Whether or not sphingolipids are scavenged remains unknown, and if *de novo* synthesis is non-essential then the status of the parasite pathway as a drug target can be in doubt.

The ability of *T. gondii* to grow in any nucleated mammalian cell line makes it a model system to investigate the role of host cell factors in parasite proliferation (Kim and Weiss, 2008). Therefore, utilizing a CHO cell line (SPB-1) with a temperature sensitive serine palmitoyltransferase (SPT) the role of host sphingolipid biosynthesis was investigated (Chapter 4). The bacterial pathogen *Chlamydia trachomatis*, which does not synthesize sphingolipids, does not replicate in this cell line under non-permissive conditions indicating that scavenged sphingolipids are essential for growth (van Ooij *et al*., 2000). In contrast, *T. gondii* was able to proliferate in sphingolipid reduced conditions (99% depletion, van Ooij *et al*., 2000) although only at ~70% of that seen in wild type or complemented SPB-1 cells (Figure 4-2). The specific SPT inhibitor myriocin gave a similar result (Figure 4-3). This small defect was rescued by the addition of serum to the media indicating that the parasites are able to scavenge sphingolipid. However, it is likely that unlike *C. trachomatis*, *de novo* sphingolipid biosynthesis is important for proliferation indicating that it may represent a valid drug target.

This is supported by the presence of sphingolipid biosynthesis in *T. gondii* (Azzouz *et al*., 2002), and in order to investigate this pathway further the sphingolipid synthase (*Tg* SLS) was identified using a bioinformatic approach (Chapter 5). *Tg* SLS is encoded by a single copy gene and like the kinetoplastid enzymes (Denny *et al*., 2006) has the conserved domains (D 1-4) found in the SM synthase family, with D 3 and 4 containing the catalytic triad (Figure 5-3; Huitema *et al*., 2004). Also, like these other synthases (Huitema *et al*., 2004; Levine *et al*., 2000) *Tg* SLS is Golgi localized (Figure 5-13).

However, unlike the human SM synthases (Figure 6-2), *Tg* SLS was able to complement an auxotrophic yeast mutant previous used to characterize the *Leishmania* enzyme (Figure 5-4; Denny *et al*., 2006). Metabolic labeling and biochemical assay using yeast-derived microsomes indicated that *Tg* SLS is able to catalyse the production of SM and, predominately, IPC (Figures 5-5 and 5-6). However, further analyses using detergent washed (to remove endogenous substrate) microsomes only showed the production of IPC when donor (phosphatidylinositol - PI, PC or phosphatidylethanolamine - PE) and acceptor (ceramide) substrates were added (Figure 5-9). In addition, neither PC nor PE demonstrated any inhibition of PI binding to the enzyme.

These data strongly indicated that *Tg* SLS is primarily an IPC synthase and a functional orthologue of AUR1. Its role as a SM synthase remains unclear. The presence of IPC synthase activity in *T. gondii* was proposed by Sonda *et al* (2005), but conclusive proof of the presence of IPC in the parasites was not forthcoming (Welti *et al*., 2007). However, metabolic labeling of isolated parasites with fluorescent-labelled ceramide indicated the presence of IPC in *T. gondii* (Figure 5-6). This was confirmed using LC-MS (Figure 5-12) and represents the first identification of IPC in the Apicomplexa.

With the identification of *Tg* SLS and it primary product in *T. gondii* consideration was given to its potential as a drug target. Sonda *et al*., (2005) showed that Aureobasidin A (Ab A) demonstrated an anti-parasitic effect against *T. gondii in vitro.* Ab A is a cyclic depsipeptide (isolated from the fungus *Aureobasidium pullulans*) and is a known inhibitor of the fungal AUR1p (Takesako *et al*., 1991). It is a comparatively large molecule with a number of side chains, several of which are believed to be important for activity (Aeed *et al*., 2009). Confirmation of the mode of action of Ab A against *Tg* SLS could provide the opportunity to consider it in terms of drug compound. However, Ab A showed little

activity against *Tg* SLS either by diffusion assay (Figure 5-6) or in biochemical assay (Figure 5-11). Indeed, the enzyme confers Ab A resistance to *S. cerevisiae* (Figure 5-7). These data demonstrated that *Tg* SLS, whilst a functional orthologue of AUR1p, is not the target of Ab A in *T. gondii,* and the activity observed is probably due to off target effects as seen in *Trypanosoma cruzi* (Figueiredo *et al*., 2005). However, *Tg* SLS may still represent a valid target for novel antiprotozoals.

Novel antiparasitics are also very much needed for the neglected tropical diseases caused by the kinetoplastid protozoa (WHO.int). As discussed the *Leishmania* IPC synthase (*Lmj* IPCS) has already been characterized (Denny *et al*., 2006; Mina *et al*., 2010b; Mina *et al*., 2011) but the orthologues apparent in the *Trypanosoma* species were unstudied at the start of this project (Chapter 6). Again employing the auxotrophic yeast mutant, YPH499-HIS-GAL-AUR1, the two *T. brucei* and one *T. cruzi* orthologues studied were shown to be functional orthologues of AUR1p (Figures 6-3 and 6-6). *Tc* IPCS 1.2 demonstrated itself to be a conventional IPC synthase by producing only IPC in both metabolic labeling and biochemical analyses (Figures 6-7 and 6-8). These data have subsequently been confirmed by others (Sevova *et al*., 2010), and in this it resembled *Leishmania* (Denny *et al*., 2006) which, like *T. cruzi,* is an intracellular pathogen.

In contrast, both *Tb* SLS orthologues examined from the extracellular pathogen *T. brucei* were able to synthesize both IPC and SM, and *Tb* SLS 4 putatively mediated the production of EPC (Figures 6-4 and 6-5). However, subsequent analyses using the microsomal system described here have only been able to demonstrate IPC synthase activity, although both putative alternative donor substrates (PC and PE) inhibit this activity indicating that they are binding to the same site on the enzyme (Mina *et al*., 2009).

This dual functionality reflects the developmental changes observed in the sphingolipid profile of *T. brucei* in differentiation from the procyclic (PCF) to the bloodstream forms (BSF) (Sutterwala *et al*., 2008). Interestingly, whilst IPC predominates in PCFs, SM does so in BSFs (Sutterwala *et al*., 2008). It may be speculated that this change from a non-mammalian sphingolipid (IPC) content towards a mammalian (SM) one help this extracellular parasite evade the host immune system.

Alternatively, IPC may have an important role to play in resistance to degradative processes in both the insect gut and in the vacuoles harbouring *Leishmania, T. cruzi* and *T. gondii*. A similar role for IPC has been proposed in *Cryptococcus neoforms*, pathogenic fungi able, like *Leishmania,* to proliferate within macrophages (Luberto *et al*., 2001). Either way, *Tb* SLS and *Tc* IPCS may prove to be valid drug targets, indeed interference RNA has been used to demonstrate this in *T. brucei* (Sutterwala *et al*., 2008; Mina *et al*., 2009). In addition, diffusion assay (Figure 6-11) and biochemical analyses (Mina *et al*., 2009) showed *Tb* SLS 4 to, like yeast, be inhibited by Ab A. Furthermore, the antifungal demonstrated submicromolar efficacy against BSF *T. brucei in vitro* (Mina *et al*., 2009). Analyses in *L. major* have shown genetically that *Lmj* IPCS is also an essential protein (Denny, personal communication). These data indicated that the *T. brucei* and *Leishmania* enzymes represent novel drug targets and, by extension supported this to be the case for orthologues in *T. cruzi* and *T. gondii*.

The Ab A sensitivity of *Tb* SLS 4 represents the exception in the Kinetoplastida (Denny *et al*., 2006; Mina *et al*., 2009; Figure 6-11) and the molecular basis of Ab A sensitivity or resistance remains unknown. However, the *Arabidopsis* IPC isoforms isolated here as functional orthologues of AUR1p (Figure 6-9) are all Ab A resistant, by diffusion assay (Figure 6-11) or biochemical assay (Mina *et al*., 2010 a). In contrast, the IPC synthase

activity from bean extracts was shown to be acutely Ab A sensitive (Bromely *et al*., 2003) as the orthologue from rice (*Oryza sativa*; Denny, personal communication), indicating that the Ab A resistance / sensitivity profiles of related organisms may be complex. In addition, like the protozoan enzymes, the plant IPC synthase has now been shown to be essential for growth and may represent a novel herbicide target (Wang *et al*., 2008).

Although the products of the sphingolipid synthases vary between Kingdoms (mammals vs fungi) and even genera (*T. brucei* vs *T. cruzi*) the mechanism of action of this expanding family of enzymes is conserved and indicates a common ancestor. Presumably it is the binding of substrates that dictates function (Mina *et al*., 2011). In keeping with this relatedness, phylogenetic analyses (Chapter 7) of various members of this family groups them into 5 clades: Animalae, Fungi, Plantae, Trypanosomatidae and Apicomplexa (Figure 7-2). The Apicomplexa represent a new group within this family and tree most closely with the Animalae and Plantae, perhaps reflecting their evolutionary origin although support for this is weak.

SUMMARY

The yeast *Saccharomyces cerivisiae* has proven to be an excellent tool to study basic cellular processes (Ko, Cheah and Fischl, 1994). In this study the engineered yeast strain YPH499-HIS-GAL-AUR1 (Denny *et al*., 2006) was used to isolate and then study the sphingolipid synthases from the model apicomplexan parasite *T. gondii;* the kinetoplastid pathogens *T. cruzi* and *T. brucei;* and the model plant *A. thaliana.* Collectively these represent promising drug and herbicide targets for future investigations and the use of a transgenic yeast system will allow the production of cost effective material for, for example, the screening of large compound libraries.

CHAPTER 09

FUTURE WORK

9 FUTURE WORK

Despite efforts to develop an effective anti-*T. gondii* vaccine, chemotherapy remains the only treatment for *T. gondii* infections (Striepen *et al*., 2000). A key factor in developing specific anti-*T. gondii* treatments is the identification of essential metabolic pathways that are restricted to the parasite and can be targeted with little or no mammalian toxicity. My results indicate that the characterization of the sphingolipid biosynthetic pathway of *T. gondii,* and the *Tg* SLS in particular, may lead to the rational development of new compounds as promising candidates for anti-apicomplexan treatments.

Tg SLS is an aureobasidin A resistant IPC synthase, however it also synthesized a minor product that co-migrates with SM. Due to the very low concentration of this species, visualisation and quantification after sphingomyelinase treatment were not successful. Therefore, employing more sensitive techniques such as mass spectrometry would be advantageous. However, *Tg* SLS remains a promising target and other further analyses are recommended:

9.1 KINETIC ANALYSES

Determination of kinetic parameters is recommended to characterise *Tg* SLS in terms of substrate binding and activity. Similar studies with *Lmj* IPCS have shown the enzyme employs a ping-pong mechanism with ceramide being the rate limiting substrate (Mina *et al*., 2010 b). This knowledge has been used to probe the possibility of inhibiting the enzyme with ceramide analogues (Mina *et al*., 2011). The materials generated and knowledge gained in these studies should be used to confirm *Tg* SLS has a similar

mechanism of action and to consider the possibility of developing rationally designed inhibitors.

9.2 SITE DIRECTED MUTAGENESIS (SDM)

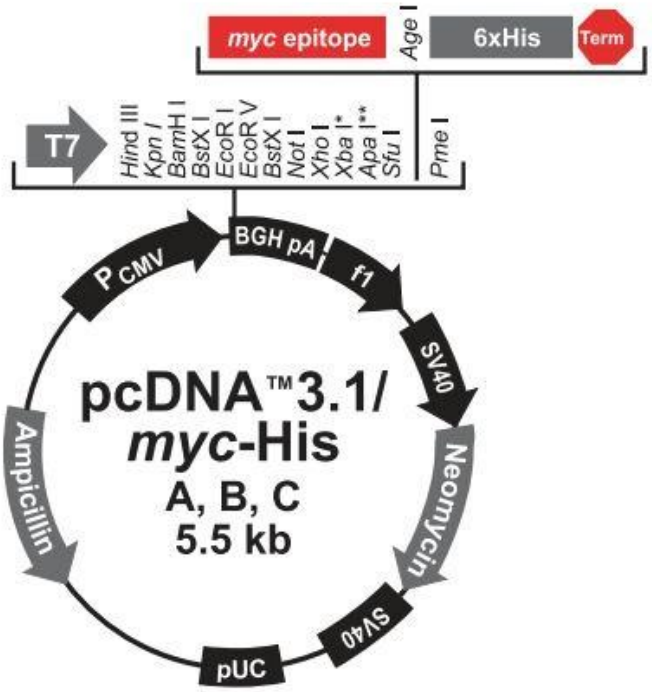
The biochemical study proposed above should be complemented using SDM to confirm that the catalytic triad, composed of two histidines and an arginine in the D3 and D4 domains, is essential for activity. Mutation of the histidine in D4 has been shown to inactivate the yeast AUR1 (Levine *et al*., 2000) and *Lmj* IPCS (Mina and Denny, personal communication). Changing this residue in *Tg* SLS, to alanine for example, should be considered. The ability of this to complement the mutant yeast could then be investigated.

9.3 ANALYZING *TG* SLS IN AN IPC SYNTHASE NEGATIVE BACKGROUND

More sophisticated analyses of mutated *Tg* SLS could be provided by expressing the enzyme in an IPC synthase null cell, rather than relying on a negative phenotype, death, in a complementation assay. Two approaches are suggested: expression in mammalian cells or in sphingolipid deficient mutant yeast (Nagiec *et al*., 2007).

pc DNA 3.1/Myc-His (+) A, B, C are vectors designed for high-level expression, purification and detection of recombinant proteins in a wide range of mammalian hosts (Figure 9-1; invitrogen.com).

Figure 9-1: pcDNA 3.1/myc-His vector with multiple cloning site and applicable restriction enzymes



Cloning the *Tg* SLS in to pcDNA 3.1/Myc-His vector and transfecting it into a mammalian cell line would allow expression levels of mutant or wild type *Tg* SLS to be assessed via the epitope tag and Western blotting, and the activity profile of the enzyme to be assessed by metabolic labeling with NBD C_6 ceramide. This would facilitate IPC synthase activity to be analyzed, but any SM (or EPC) synthase activity could be masked by the endogenous activity of the mammalian cell.

AGD yeast lack a functional AUR1p and SPT, and they survive by making unusual phospholipids (Nagiec *et al*., 1997). Expression of *Tg* SLS in these mutants would allow the analysis of the enzyme in a cell background free from sphingolipid biosynthesis. However, in keeping with the observations of Nageic *et al*., (2007) transformation of AGD has proven difficult and this approach has yet to prove successful (Mina and Denny, personal communication).

9.4 *TG* SLS KNOCK-OUT

Unequivocal proof that *Tg* SLS is a valid drug target will come from the analyses of transgenic *T. gondii* in which *Tg* SLS is deleted via homologous recombination. *T. gondii* represents an excellent system for these kinds of experiments due to the array of genetic tools available. For example if, as expected, *Tg* SLS represents an essential activity, the tetracycline trans-inducible system utilization could be used (Meissner *et al*., 2007). In this protein expression is placed under the control of an inducible promoter so that it can be down regulated on the addition of tetracyclin during the course of an assay.

These analyses are essential to demonstrate whether *Tg* SLS is essential and so a drug target, they will also indicate the role of the enzyme and sphingolipids in parasite proliferation and pathogenesis.

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APPENDICES

APPENDIX (I)

STANDARD PROTOCOLS

1.1 PCR

1.1.1 Amplification of the gene of interest with proof reading enzyme

Template DNA	0.5 µl (0.5-1.0µg/ml)	
3’ Primer	1 µl (100µM)	
5’ Primer	1 µl	
2.5mM dNTP	5 µl	
10X P*fu* buffer	5 µl	
P*fu* DNA polymerase	1 µl	
Nuclease free water	36.5 µl	for the final volume of 50 µl

PCR program was set as,

95 °C 2 minutes (fin hold)

30 cycles

95 °C 30 seconds

55 °C 30 seconds (annealing)

72 °C 2 minute 20 seconds (extension)

72 °C 5 minutes (final extension)

4 °C hold

1.1.2 Amplification of the gene of interest with GoTaq polymerase

Go *Taq* green master mix	12.5 µl	
3’ primer	0.5 µl	
5’ primer	0.5 µl	
Template DNA	2.5 µl	
Nuclease free water	9 µl	for the final volume of 25 µl

PCR program was set as,

95 °C 2 minutes (fin hold)

30 cycles

95 °C 30 seconds

55 °C 30 seconds (annealing)

72 °C 1 minute 10 seconds (extension)

72 °C 5 minutes (final extension)

4 °C hold

1.1.3 Amplification of the gene of interest with FideliTaq polymerase

Template DNA	2 µl	
3’ primer	0.5 µl	
5’ primer	0.5 µl	
10mM dNTP	1 µl	
10X PCR Buffer	5µl	
FideliTaq DNA polymerase	0.5µl	
Nuclease free water	40.5 µl	for the final volume of 50 µl

PCR program was set as,

95 °C 2 minutes (fin hold)

30 cycles

94 °C 30 seconds

55 °C 30 seconds (annealing)

68 °C 1 minute 30 seconds (extension)

68 °C 5 minutes (final extension)

4 °C hold

1.2 DIGESTION OF A PCR PRODUCT

1.2.1 For specific restriction enzyme in a specific buffer

DNA	20 µl	
Nuclease free water	6.8 µl	
10X Buffer H	2 µl	
10mg/ml BSA	0.2 µl	
Restriction enzyme	1 µl	(final volume of 30 µl)

Incubated at 37 °C for 2 hours in Perkin Elmer DNA Thermal Cycler

1.2.2 In the presence of a common buffer

DNA	15.8 µl	
Restriction enzyme I	1 µl	
Restriction enzyme II	1 µl	
10X Buffer	2 µl	
10mg/ml BSA	0.2 µl	(final volume of 20 µl)

Incubated at 37 °C overnight in Perkin Elmer DNA Thermal Cycler

1.3 LIGATION OF DNA IN TO A VECTOR

1.3.1 Ligation using Perkin Elmer DNA Thermal Cycler

Purified digested DNA	7 µl
Purified digested vector	1 µl
10X Ligase buffer	1 µl
3 µ/µl DNA ligase	1 µl

Incubated at 14 °C overnight in Perkin Elmer DNA Thermal Cycler

1.3.2 Ligation using PCR programmer

Purified digested DNA	2 µl (or water for control)
Purified digested vector	10 µl

Incubated at 65 °C for 15 minutes at the PCR programmer. When the temperature dropped down to 18-20 °C, the program was paused and the followings were added.

Ligase buffer	1 µl
T4 DNA ligase	1 µl

Incubated at 16 °C for 30 minutes for the extension and fin hold at 4 °C

1.4 PREPARATION OF GLYCEROL STOCKS

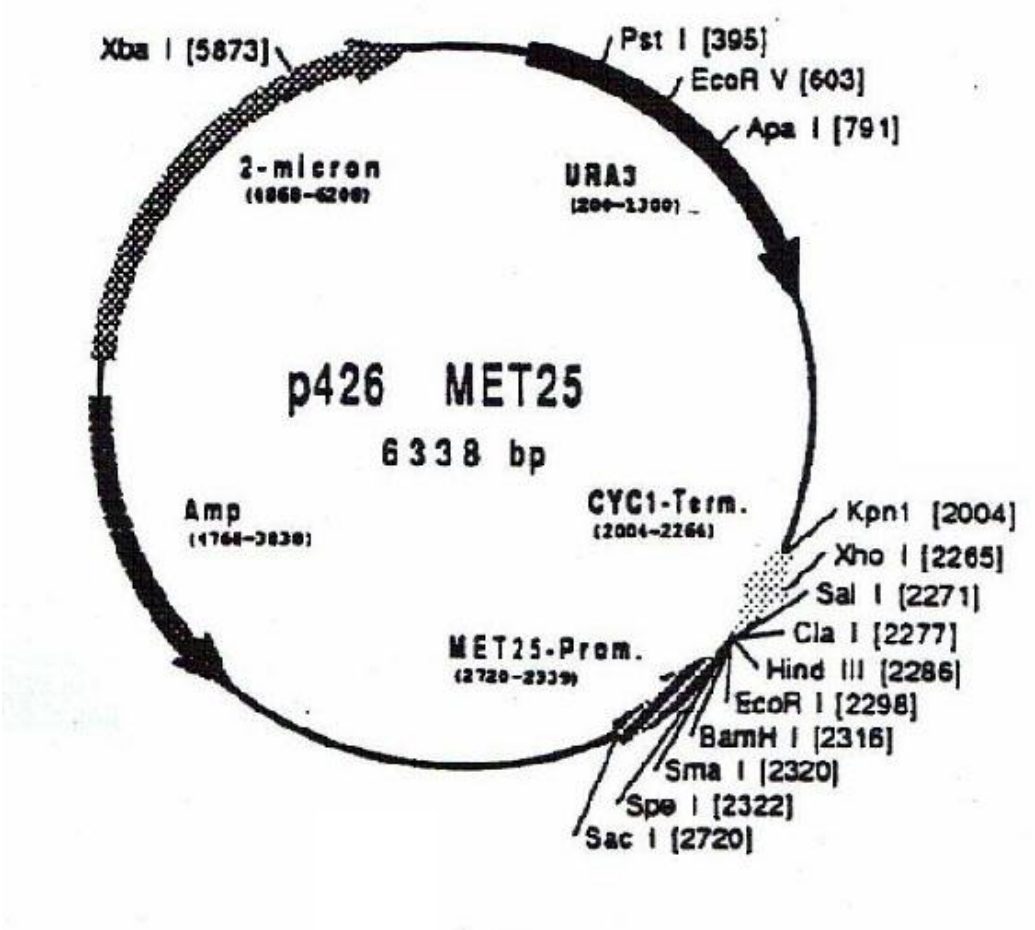
500 µl of purified mini prep sample or cell culture were transferred in to a sterile screw cap micro centrifuge tube. 500 µl of 80% glycerol were added and vortexed. This was stored at -80 °C for until further use.

1.5 NBD C_6 CERAMIDE AND BSA CONJUGATION

1 mg of NBD C_6 ceramide was dissolved in 1.740 ml of Chloroform: ethanol (19:1 v/v) and 1 mM of NBD C_6 ceramide stock was prepared. 50 µl of 1 mM stock was concentrated in a 1.5 ml LoBind® Eppendorf tube for 1 hour at 35 °C. Dried pellet was re-suspended in 200 µl of absolute ethanol. 10 ml of DMEM without BSA was filter sterilized and 3.4 mg of de-fatted BSA were added and mixed. 200 µl of NBD C_6 ceramide dissolved in ethanol was pipetted in to 10 ml of DMEM added with defatted BSA while mixing on a vortex mixture. The resulted solution of 5 µM NBD C_6 ceramide: 5 µM BSA was aliquoted as 1 ml in LoBind® Eppendorf tubes and stored at -20 °C.

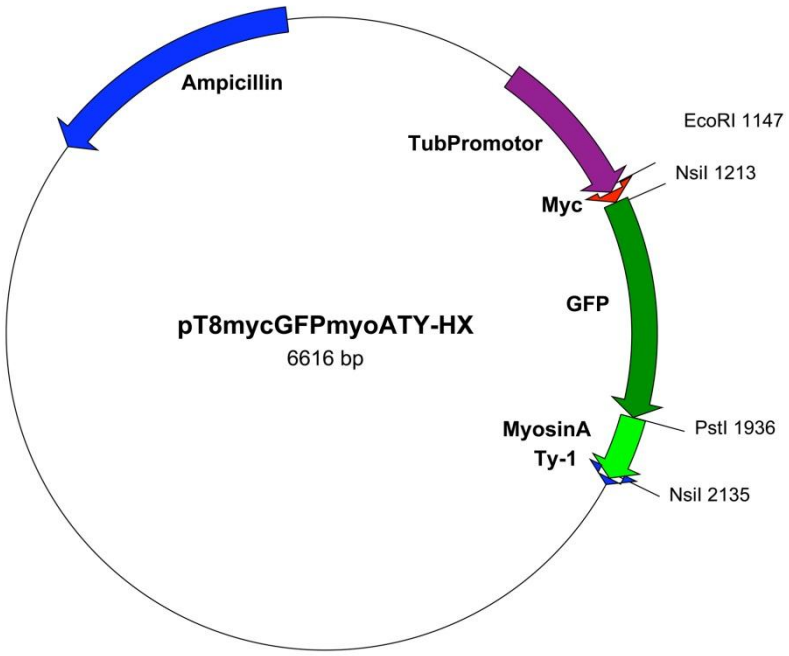
APPENDIX (II)

pRS 426 MET 25 vector



APPENDIX (III)

pT8MycTy vector



RESEARCH PUBLICATIONS

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The *Trypanosoma brucei* sphingolipid synthase, an essential enzyme and drug target☆

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ABSTRACT

Sphingolipids are important components of eukaryotic membranes, particularly the plasma membrane, and are involved in a diverse array of signal transduction processes. In the Eukaryota the biosynthetic pathway for the formation of these lipid species is largely conserved. However, in contrast to mammals which produce sphingomyelin (SM), several pathogenic fungi and protozoa synthesize inositol phosphorylceramide (IPC) as the primary phosphosphingolipid. This process is catalyzed by the enzyme IPC synthase, a recognized target for anti-fungals encoded by the *AUR1* gene in yeast. Recently, functional orthologues of the AUR1p have been identified in a group of insect vector-borne pathogenic protozoa, the Kinetoplastida, which are responsible for a range of so-called neglected diseases. Of these the *Trypanosoma brucei* species are the causative agents of human African trypanosomiasis in many of the most under-developed regions of Africa. The available treatments for these diseases are limited, of decreasing efficacy, and often demonstrate severe side-effects. Against this background the *T. brucei* sphingolipid synthase, an orthologue of the yeast AUR1p, may represent a promising target for novel anti-protozoals. Our studies identify an isoform of this protein as a novel bi-functional enzyme capable of catalyzing the synthesis of both IPC and SM, both known to be present in the parasite. Furthermore, the synthase is essential for parasite growth and can be inhibited by a known anti-fungal at low nanomolar levels *in vitro*. Most notably this drug demonstrates trypanocidal activity against cultured bloodstream form parasites. Thus, the *T. brucei* sphingolipid synthase represents a valid and promising drug target.

1. Introduction

Trypanosoma brucei species are protozoan parasites of the order Kinetoplastidae and the etiological agents of both human African trypanosomiasis (HAT, sleeping sickness), and diseases of economically important animals (e.g. nagana in cattle) [1]. These diseases are endemic in much of sub-Saharan Africa, with HAT causing a burden of approximately 1.6 million disability adjusted life years (http://www.who.int/tdr/). This distribution across some of the most under-developed regions of the world is coupled with a paucity of effective therapies, with those available being either too expensive (e.g. eflornithine) or exhibiting catastrophic side-effects (e.g. melarsoprol). Together with the leishmaniases (caused by the related *Leishmania* species) HAT has been described as an emerging or uncontrolled disease. Therefore, there is an urgent need for new, validated drug targets and anti-HAT compounds to combat a disease causing in excess of 50,000 deaths per annum [2].

Sphingolipids are a diverse group of amphipathic lipids that perform essential functions in eukaryotes. For example, the unmodified sphingolipid ceramide acts as a secondary signalling molecule [3] and more complex species are implicated in the formation and function of signal transduction complexes [4,5]. The primary phosphosphingolipid species in mammalian species, including humans, is sphingomyelin (SM). SM is formed by the transfer of the phosphorylcholine head group from phospholipid phosphatidylcholine (PC) to ceramide, a reaction catalyzed by SM synthase [6]. In contrast

☆ Since the initial submission of this work to Molecular and Biochemical Parasitology a paper has been published in Molecular Microbiology identifying and characterising the same family of enzymes in *Trypanosoma brucei*: Sutterwala S.S., Hsu F.F., Sevova E.S., Schwartz K.J., Zhang K., Key P., Turk J., Beverley S.M., Bangs J.D. Developmentally regulated sphingolipid synthesis in African trypanosomes (2008) 70:281–296.

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fungi, plants and at least some protozoa produce inositol phosphorylceramide (IPC) as their primary phosphosphingolipid [7]. In these organisms IPC synthase catalyzes the transfer of phosphorylinositol from phosphatidylinositol (PI) to ceramide [8–10]. IPC synthase has long been established and studied as a target for novel anti-fungals [11,12]. More recently this enzyme has come under scrutiny as a potential target for anti-protozoals [13]. With the recent identification and characterization of the *Leishmania* IPC synthase (LmIPCS; [10]) it has become possible both to validate this protozoan activity as a drug target and, furthermore, begin to investigate potential inhibitors, including those known to act against the fungal IPC synthase. Four closely related orthologues of LmIPCS are apparent in the *T. brucei* database [10], and mass spectrometry of isolated fractions has revealed that whilst the predominant phosphosphingolipid in pathogenic bloodstream form parasites is SM [14]; insect stage, procyclic *T. brucei* also contain IPC [15].

Here we describe the characterization of the African trypanosome, *T. brucei*, sphingolipid synthase 4 (TbSLS4) which demonstrates itself to be a novel bi-functional enzyme with the ability to catalyze the biosynthesis of both IPC and SM, thus reflecting the sphingolipid profile of the parasite. Importantly, the IPC synthase activity of TbSLS4 is acutely sensitive to the well characterized specific fungal inhibitor aureobasidin A [11,12] and pathogenic bloodstream form *T. brucei* are rapidly killed at sub-micromolar concentrations of this drug. Furthermore, down-regulation of TbSLS1–4 using inhibition RNA (RNAi) in bloodstream form parasites demonstrated that the enzyme activity is essential for growth thus validating it as a target for the development of new anti-HAT therapies.

2. Materials and methods

2.1. Functional identification of the T. brucei sphingolipid synthase

A common, conserved AUG start codon was predicted for all four TbSLS isoforms by examination of the genome sequence (www.genedb.org). Subsequently, TbSLS1 (Tb09211.1030) and TbSLS4 (Tb09211.1000) were amplified with *Pfu* polymerase (Promega) from *T. brucei* strain Lister 427 genomic DNA using primer pairs (homologous sequence underlined): TbSLS1—CCGGAATTCATGATTAGTTACCCTTTCTTCTCCC and CCGCTCGAGTCATACCTCGTTAGTTGATAC; TbSLS4—CCGGAATTCATGATTAGTTACCCTTTCTTCTCCC and CCGCTCGAGTCACACATACGCCCCACATTTAAAC;

The PCR products were subsequently cloned into the yeast expression vector pRS426 MET [16] to give pRS426 TbSLS1 and pRS426 TbSLS4. These, together with pRS426 AUR1, pRS426 human sphingomyelin synthase 1 and 2 (HsSMS1 and 2) and empty vector (pRS426), were used to transform the YPH499-HIS-GAL-AUR1 *Saccharomyces cerevisiae* strain [10]. Transformants were selected on non-permissive SD medium (0.17% Bacto yeast nitrogen base, 0.5% ammonium sulphate and 2% dextrose) or permissive SGR medium (0.17% Bacto yeast nitrogen base, 0.5% ammonium sulphate, 4% galactose and 2% raffinose) containing the appropriate nutritional supplements at 30 °C.

2.2. Metabolic labelling and analyses

Yeast were grown to exponential phase in SD or SGR as indicated and 2.5 OD_{600} units incubated in 1 ml of SD or SGR supplemented with 5 μM of NBD C_6-ceramide (Invitrogen) conjugated to fat-depleted bovine serum albumin (Sigma–Aldrich) for 120 min at 30 °C. Yeast were harvested by centrifugation and washed twice with phosphate buffered saline. Chloroform/methanol (0.4 ml; 1:1, v/v) was added and cells were disintegrated with glass beads. The pellet was re-extracted with chloroform/methanol/water (10:10:3) and the lipid fraction isolated by phase separation. After drying in a rotavapor (Eppendorf Concentrator 5301) reaction products were re-suspended in 20 μl of 10:10:3 and cell mass equivalents fractionated using HPTLC silica plates (Merck) and the eluent system chloroform:methanol:aqueous 0.25% KCl (55:45:10). Imaging and quantification was carried out using a FLA3000 scanner (Fujifilm) and AIDA Image Analyzer® software (version 1.3). Vero cells were labelled and processed for use as controls and standards as previously described [10].

Sphingomyelinase (*Bacillus cereus*, Sigma–Aldrich) was used to identify sphingomyelin in the NBD C_6-ceramide lipids extracted from the complemented yeast as previously described [17]. Briefly, YPH499-HIS-GAL-AUR1 *S. cerevisiae* complemented with pRS426 TbSLS4 (in SD) or transformed with pRS426 HsSMS2 or empty vector (in permissive SGR, or for a limited period SD) were grown to exponential phase. After adjustment to an optical density of 0.5 OD_{600} in 5 ml of either SD or SGR, cells were labelled for 16 h with 2 μM of NBD C_6-ceramide conjugated to fat-depleted bovine serum albumin as previously described [6]. Labelled lipid fractions from these were prepared and dried as above with 50 μg of sphingomyelin (Sigma–Aldrich), and re-suspended, with sonication in a water bath, in 600 μl of 20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$ and 0.05% (w/v) Triton X-100. Subsequently, samples (300 μl) were incubated with or without 2 units of *B. cereus* sphingomyelinase (Sigma–Aldrich) at 37 °C for 120 min. Equivalent lipid extracts were fractionated and analyzed as above.

2.3. In vitro assay of TbSLS4 activity

Microsomal membranes from exponentially growing YPH499-HIS-GAL-AUR1 pRS426 TbSLS4, pRS426 LmIPCS or pRS426 AUR1 [10] were prepared as previously described [18] 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. The microsomal membranes were subsequently washed in 2.5% CHAPS (w/v; Sigma–Aldrich; 4 °C, 60 min), isolated by centrifugation (150,000 × *g*, 4 °C and 90 min), re-suspended in storage buffer at 10 mg/ml and stored at −80 °C until use.

The assay mix contained 100 μM donor substrate (bovine liver PI, PC or PE, Avanti Polar Lipids), 10 μg of microsomes, 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). After biphasic separation the organic layer was removed, processed and analyzed as above.

For inhibition experiments the reaction mix was pre-incubated for 30 min with appropriate quantities of aureobasidin A (Takara) before the addition of NBD C_6-ceramide.

2.4. Parasite culture

Bloodstream form *T. brucei* strains Lister 427 and its engineered variant, Single Marker Bloodstream form (SMB, T7RNAP::TETR::NEO; [20]) were maintained *in vitro* at 37 °C with 5% CO_2 in HMI-9 medium supplemented with 10% FCS and, for SMB, 2.5 μg/ml G418.

2.5. Inhibition RNA (RNAi) of TbSLS

An 165 base pair sequence fragment common to all four TbSLS open reading frames was amplified from genomic DNA using *Pfu* polymerase and primer pair (homologous sequence underlined): CATAGATCTAGAGGTTCCATACACTGTG and CATAGATCTAGACGAGAGGCAACGATGC.

This PCR product was cloned into the RNAi vector p2T7 [21] and, following linearization, 10 μg transfected into SMB *T. brucei* and

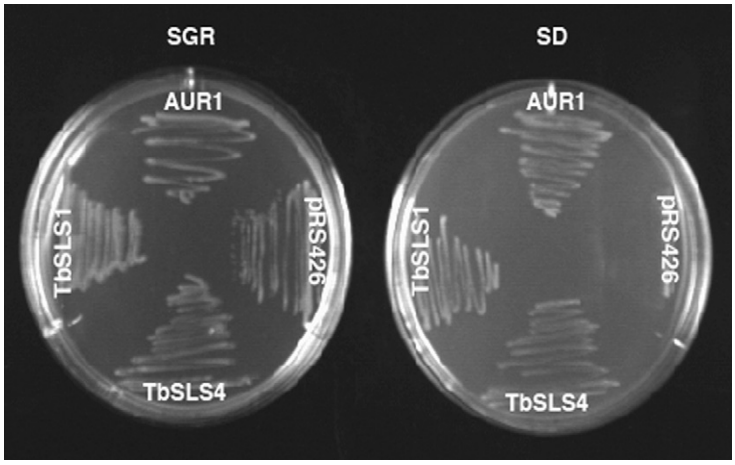

Fig. 1. Transformation with pRS426 TbSLS4 and pRS426 TbSLS1, as well as pRS426 AUR1 (the *S. cerevisiae* IPC synthase), rescues the auxotrophic mutant YPH499-HIS-GAL-AUR1 allowing it to grow in the presence of glucose (SD media). YPH499-HIS-GAL-AUR1 pRS426 (empty vector) does not grow in the presence of glucose (SD), however all lines are viable in the absence of glucose and the presence of galactose (SGR).

transformants selected using 2 μg/ml phleomycin (Sigma–Aldrich). Following induction with 1 μg/ml doxycycline cell growth was determined at 24 h intervals by light microscopy using an Improved Neubauer Haemocytometer.

48 h post-induction total RNA was isolated (RNeasy®, Qiagen) and RT-PCR was performed (SuperScript II, Invitrogen) using the primer pairs:

TbSLS: AAACTGTACCTTCTTCACCG and CGAGAGGCAACGATGC;
Tb β tubulin: GGAGCGCATCAATGTGTAC and CAGGCAGCAGGTGACGCCG.

2.6. *T. brucei* susceptibility to aureobasidin A

T. brucei Lister 427 were cultured in the presence of various concentrations of aureobasidin A. Growth was analyzed at 24 h intervals as above.

3. Results and discussion

3.1. Identification and characterization of the *T. brucei* sphingolipid synthase

Four tandem gene sequences (Tb09211.1030, Tb09.211.1020, Tb09.211.1010 and Tb09.211.1000; here annotated TbSLS1–4 to reflect their 5′–3′ order) were previously identified in the *T. brucei* genome database (www.genedb.org) as sequence orthologues of the inositol phosphorylceramide synthase (LmIPCS) from the related kinetoplastid parasite, *Leishmania* [10]. The predicted open reading frames encode four closely related trans-membrane proteins with more than 90% identity and 94% similarity. Most variation occurs at the carboxy-termini, a region predicted to lie on the cytosolic side of the membrane away from the active site at the Golgi lumen, with another variable domain close to and within the second predicted trans-membrane domain [10]. One of the predicted *T. brucei* sphingolipid synthase isoforms (Tb09.211.1000; TbSLS4) was the focus of this study. In addition, the isoform most distant from TbSLS4 with respect to the internal variable domain (Tb09.211.1030; TbSLS1) was also subjected to preliminary analyses.

The auxotrophic mutant *S. cerevisiae* strain, YPH499-HIS-GAL-AUR1, has the essential *AUR1* IPC synthase gene under the control of a galactose inducible promoter. Therefore it is unable to grow in the presence of the repressor glucose, a phenotype that is rescued by the ectopic expression of LmIPCS [10]. Similarly, TbSLS4 and TbSLS1 expression complemented the YPH499-HIS-GAL-AUR1 mutant yeast line indicating that they are also functional orthologues of the yeast *AUR1* gene (Fig. 1). The yeast IPC synthase (AUR1) also complemented this mutant line. Significantly, neither HsSMS1 nor HsSMS2 complemented the YPH499-HIS-GAL-AUR1 yeast (data not shown) indicating that sphingomyelin synthase activity alone is not sufficient to rescue the mutant.

To understand the function of the *T. brucei* sphingolipid synthase, the auxotrophic YPH499-HIS-GAL-AUR1 yeast cells complemented with either TbSLS4, TbSLS1 or yeast AUR1 were metabolically labelled with fluorescent NBD C_6-ceramde, a substrate for sphingolipid synthases, including those from the kinetoplastids [10]. Under the conditions described, the AUR1 complemented *S. cerevisiae* auxotrophic mutant synthesized IPC as the only labelled product. In contrast, both TbSLS4 and TbSLS1 complemented mutant yeast were shown to synthesize two major labelled lipid species. One of these co-migrated with SM, the other with IPC. The latter at levels equivalent to those produced in the AUR1 complemented yeast (Fig. 2A). In addition, TbSLS4 complemented yeast synthesized a third species which co-migrated with an unknown lipid (X) produced by labelled mammalian cells (Vero). As a control YPH499-HIS-GAL-AUR1 cells harbouring an empty vector (pRS426) were cultured in both permissive (SGR) and non-permissive (SD) media. Both lines grew equivalently in both media for 16 h and for a further 8 h after dilution to an optical density 0.3 OD_{600}, and remained viable (by plating on permissive media, data not shown). Labelling of these dividing cells with NBD C_6-ceramde in their respective media under the same conditions as above demonstrated that in non-permissive SD no labelled IPC is produced, indicating the down-regulation of AUR1p (Fig. 2B).

TbSLS4 was chosen for further study due to the relative predominance of the SM-like species in the labelled complemented yeast. Unlike IPC, SM is known to be present in the pathogenic bloodstream form of the parasite [14]. The labelled SM-like species (and the unknown X) proved to be susceptible to sphingomyelinase (which breaks down SM into phosphorylcholine and ceramide) when lipid extracts were treated with this enzyme, thereby confirming its identity (Fig. 3A). In contrast the IPC produced was insensitive to this enzyme treatment. Extracts from the auxotrophic mutant expressing HsSMS2, which produce an equivalent quantity of labelled SM under permissive conditions (SGR), acted as a control for SMase activity.

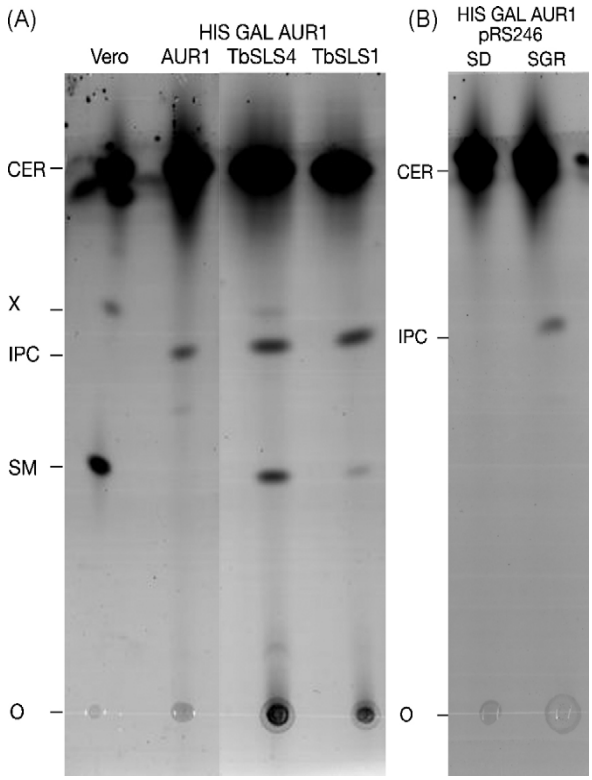

Fig. 2. (A) Metabolic labelling of yeast IPC synthase (AUR1) complemented YPH499-HIS-GAL-AUR1 with NBD C_6-ceramide in glucose-containing media (SD) showed that they synthesize only labelled IPC. In contrast the same mutant line, labelled under the same conditions, but complemented with TbSLS4 or TbSLS1 synthesized two predominant sphingolipid species, one of which co-migrated with IPC, the other with SM. In addition, in TbSLS4 complemented yeast a minor labelled species (X) was evident which co-migrated with an unknown detected in labelled mammalian cell (Vero) extracts, in which the predominant complex sphingolipid is SM. (B) The yeast IPC synthase (AUR1) is down-regulated in YPH499-HIS-GAL-AUR1 cells transformed with empty vector (pRS426) when incubated in non-permissive, glucose-containing media (SD). This is demonstrated by the lack of detectable IPC in yeast metabolically labelled with NBD C_6-ceramide in SD under the same conditions as above. In galactose-containing, glucose-free media (SGR) the synthesis of IPC is clearly evident. NBD C_6-ceramide labelled lipid extracts fractionated by HPTLC, representative of at least three independent experiments. O, origin; IPC, inositol phosphorylceramide; SM, sphingomyelin; Cer, ceramide (migrating at the front); X, unknown sphingolipid. All lipid extracts normalised with respect to cell mass.

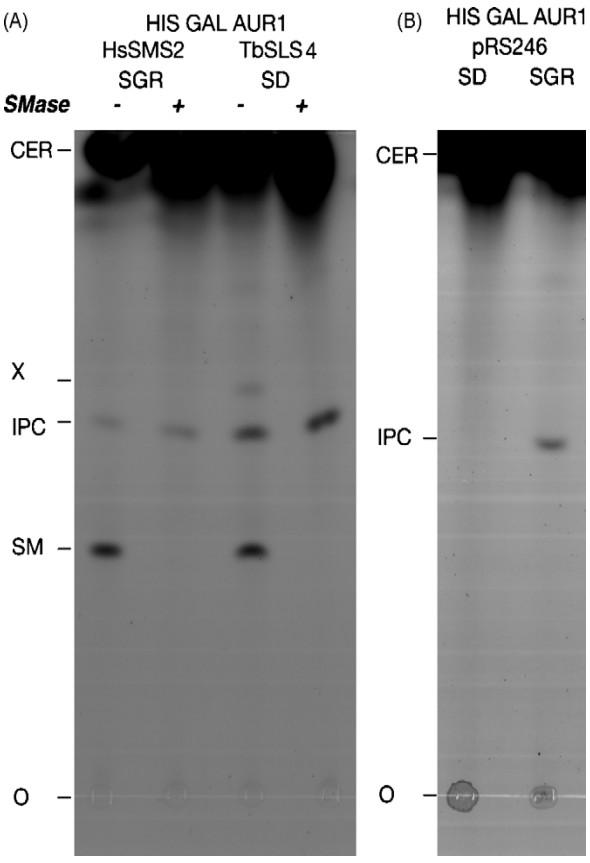

Fig. 3. (A) Sphingomyelinase (SMase) treatment of lipids extracted from YPH499-HIS-GAL-AUR1 complemented with TbSLS4, grown in glucose-containing media (SD) and labelled with NBD C_6-ceramide, demonstrated that the predicted sphingomyelin species is SMase sensitive, as is unknown X. In contrast the predicted IPC is insensitive. As a control, YPH499-HIS-GAL-AUR1 expressing human SM synthase 2 (HsSMS2, grown in permissive SGR media) was utilized as they produce equivalent quantities of labelled SM to the TbSLS4 line. (B) The yeast IPC synthase (AUR1) is down-regulated in YPH499-HIS-GAL-AUR1 cells transformed with empty vector (pRS426) incubated in non-permissive SD under the same conditions as the SMase treated samples. This is demonstrated by the lack of detectable IPC in yeast metabolically labelled with NBD C_6-ceramide in SD. In galactose-containing, glucose-free media (SGR) the synthesis of IPC is clearly evident. NBD C_6-ceramide labelled lipid extracts fractionated by HPTLC, representative of at least three independent experiments. O, origin; IPC, inositol phosphorylceramide; SM, sphingomyelin; Cer, ceramide (migrating at the front); X, unknown sphingolipid. All lipid extracts normalised with respect to cell mass.

Together these data suggest that TbSLS4 is a novel bi-functional enzyme acting as both a SM and an IPC synthase. This is consistent with previous analyses which demonstrated that the parasite harbours both SM [14] and IPC phosphosphingolipids [15].

3.2. *In vitro analyses of TbSLS4 activity*

To further investigate the function of TbSLS4, microsomal material was isolated from the TbSLS4 and AUR1 complemented yeast as described and used in an *in vitro* assay utilising the common acceptor substrate NBD C_6-ceramide and the candidate donor substrates bovine liver PI, PC and phosphatidylethanolamine (PE; a potential substrate for ethanolamine phorphorylceramide synthesis [22]). Notably, when assayed, the yeast AUR1 crude microsomal material showed significant IPC synthase turnover, but demonstrated little significant increase in this on the addition of the donor substrate PI. As expected, no detectable SM synthesis was observed with or without the addition of PC (Fig. 4A). In order to clearly assign enzyme function this assay was refined according to data obtained from the analysis of LmIPCS (Mina et al. in preparation). In brief, microsomal fractions were washed with ice-cold 2.5% 3-[3-(cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) to remove endogenous (i.e. yeast) substrates. This facilitated analyses of the effect of adding exogenous substrate to the reaction—in this case the candidate donor substrates (PI, PC or PE) plus the known acceptor substrate NBD C_6-ceramide. Without the addition of the donor substrate the CHAPS-washed AUR1 microsomes, compared to the crude unwashed sample, demonstrated a relatively low level of IPC synthase turnover and, as expected no evidence of SM synthase function (Fig. 4A, crude and washed). Importantly, the addition of PI or PC had no discernable effect on enzyme turnover in the washed sample indicating that the yeast IPC synthase is either substrate specific (and unable to utilize bovine PI) or that the detergent treatment had disrupted the protein (Fig. 4A, +PI and +PC washed). In contrast, assay of identically treated TbSLS4 microsomes showed that the addition of PI led to a large (more than 12-fold) increase in the formation of IPC (Fig. 4B, +PI). This demonstrated that TbSLS4 functions as an IPC synthase, an activity not attributable to background AUR1 expression.

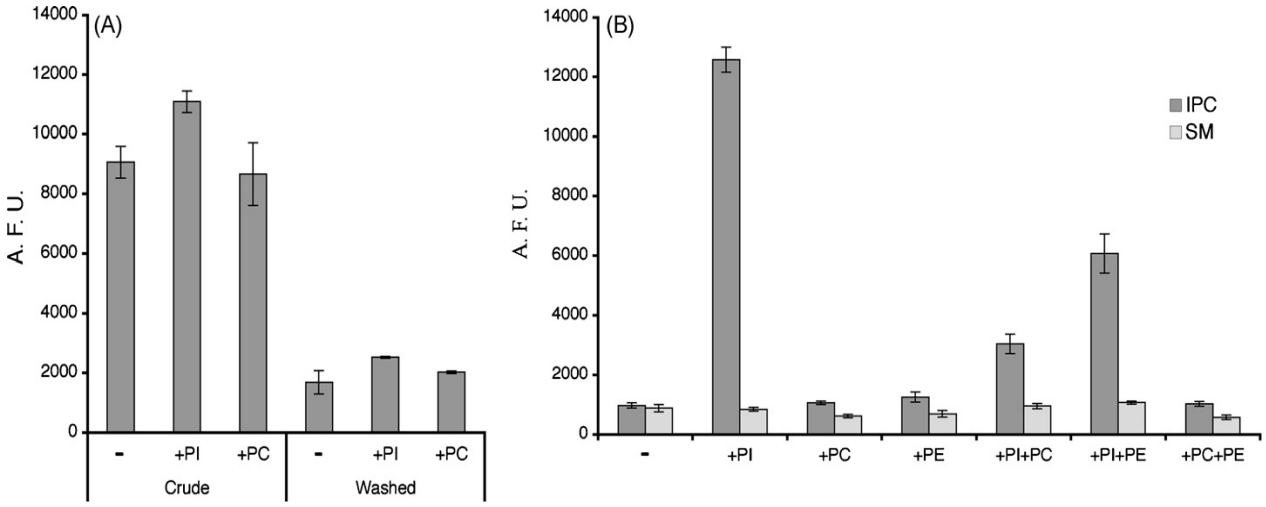

Fig. 4. Detergent-washed microsome extracts from YPH499-HIS-GAL-AUR1 TbSLS4 yeast demonstrate IPC synthase enzyme turnover on the addition of the donor substrate PI and acceptor substrate NBD C_6-ceramide, but no further production of other sphingolipid species on the addition of alternative donors PC and PE. (A) The addition of bovine liver PI led to a marginal increase in IPC production in unwashed (crude) AUR1 microsomes when compared to the control (–). In CHAPS-treated microsomes (washed) IPC synthase (AUR1) turnover was minimal without donor substrate (–) and unchanged by the addition of either PI or PC as donors. This demonstrated that the yeast IPC synthase (AUR1) was unable to utilize these donor substrates under the experimental conditions. (B) In contrast, assay of TbLS4 microsomes (CHAPS-treated, as the washed sample in A) demonstrated a greater than 12-fold increase in IPC over a sample without donor substrate (–) on the addition of PI. PC and PE had no demonstrable effect on enzyme turnover. However, both inhibited IPC synthesis when added together with PI at equivalent molar quantities: PC by approximately 4-fold and PE by approximately 2-fold. NBD C_6-ceramide labelled lipid extracts fractionated by HPTLC and quantified as described, standard deviation of three independent experiments shown. AFU, arbitrary fluorescence units; IPC, inositol phosphorylceramide; SM, sphingomyelin; –, no donor substrate added; PI, bovine liver phosphatidylinositol; PC, bovine liver phosphatidylcholine; PE, bovine liver phosphatidylethanolamine.

Surprisingly, given the identification of SM as a TbSLS4 product above (Fig. 3A), bovine PC – a potential donor substrate for a SM synthase – had no significant effect on enzyme activity (Fig. 4B, +PC). The addition of PE was similarly ineffectual (Fig. 4B, +PE). However, when PC or PE were added simultaneously as molar equivalents with PI to the assay system, the quantity of IPC produced decreased 4- and 2-fold respectively. This suggested that both PC and PE bind competitively with PI to the same region of the enzyme (Fig. 4B, +PI + PC and +PI + PE).

Taken together these results indicate that TbSLS4 functions as an IPC synthase but also binds PC (and PE) and can participate in the synthesis of SM. The lack of *in vitro* SM synthase activity in the presence of exogenous PC is surprising and may relate to substrate specificity (see yeast AUR1 and bovine PI, Fig. 4A, washed). One possibility is that TbSLS4, in this assay, is unable to utilize bovine liver PC. This donor substrate is a mixed natural product with predominantly C36:2 PC. In contrast, *S. cerevisiae*, where both TbSLS4 and TbSLS1 function as SM synthases, predominantly possesses C32:2 and C34:2 PC [23]. Perhaps TbSLS favours these relatively short acyl groups? However, *T. brucei* procyclic and bloodstream forms harbour significant quantities of C36, C38 and C40 PC species [14,24,25], indicating that any substrate selectivity is perhaps due more subtle structural differences. In support of this, although TbSLS4 can utilize bovine liver PI (predominantly C38:4) efficiently as a substrate for IPC synthesis, procyclic form parasites (known to synthesize IPC [15]) harbour only trace levels of the C38:4 PI [14].

3.3. Inhibition of TbSLS4 using a known anti-fungal agent

IPC synthase is a recognized target for anti-fungal drugs and the natural product aureobasidin A is a widely utilized and specific experimental inhibitor [26]. This drug also specifically inhibits the activity of *Leishmania* LmIPCS, a TbSLS orthologue, albeit at a concentration several orders of magnitude higher than those for the *S. cerevisiae* enzyme [10].

To establish the efficacy of aureobasidin A against the *T. brucei* enzyme the previously described *in vitro* assay was employed using CHAPS-washed TbSLS4 microsomes with NBD C_6-ceramide and bovine PI as receptor and donor substrates respectively. As a control identically prepared LmIPCS microsomes were assayed in the same manner. Given the inactivity of the yeast enzyme in this assay system it was not possible to include this as a further control. The synthesis of labelled IPC was used as a measure of IPC synthase turnover. From this assay it was evident that the *T. brucei* enzyme is acutely sensitive to the drug (Fig. 5A). Following reanalysis of the linear portion of the curve (0–0.5 nM aureobasidin A), the turnover was calculated to be 50% inhibited (IC_{50}) by 0.42 nM aureobasidin A. TbSLS4 turnover was undetectable at a concentration of 50 nm aureobasidin A. In contrast, the IC_{50} for LmIPCS inhibition by aureobasidin A was more than 200,000 times higher, with precipitation of the drug at concentrations above 100 μM (Fig. 5B) preventing the determination of an absolute value.

3.4. Validation of TbSLS as a target of anti-protozoals

Using a sequence fragment common to all four TbSLS isoforms an RNAi construct was prepared in the p2T7 vector [21] and used to specifically inhibit TbSLS expression in cultured bloodstream form *T. brucei* (SMB). Non-induced TbSLS RNAi cells grew in a similar manner to control SMB parasites carrying empty vector with or without the doxycycline. In contrast, doxycycline induction of TbSLS RNAi saw the parasites cease division and led to some cell death as scored by light microscopy. RT-PCR, using β tubulin as a control, confirmed the specificity of the TbSLS mRNA inhibition (Fig. 6).

This genetic approach validated TbSLS as an essential enzyme for pathogenic bloodstream form parasite growth. Given the *in vitro* data shown above in which aureobasidin A was demonstrated to inhibit TbSLS4, the efficacy of this natural compound was tested against cultured bloodstream form *T. brucei* (Lister 427; Fig. 7). When the concentration of aureobasidin A was 1 μM cell growth

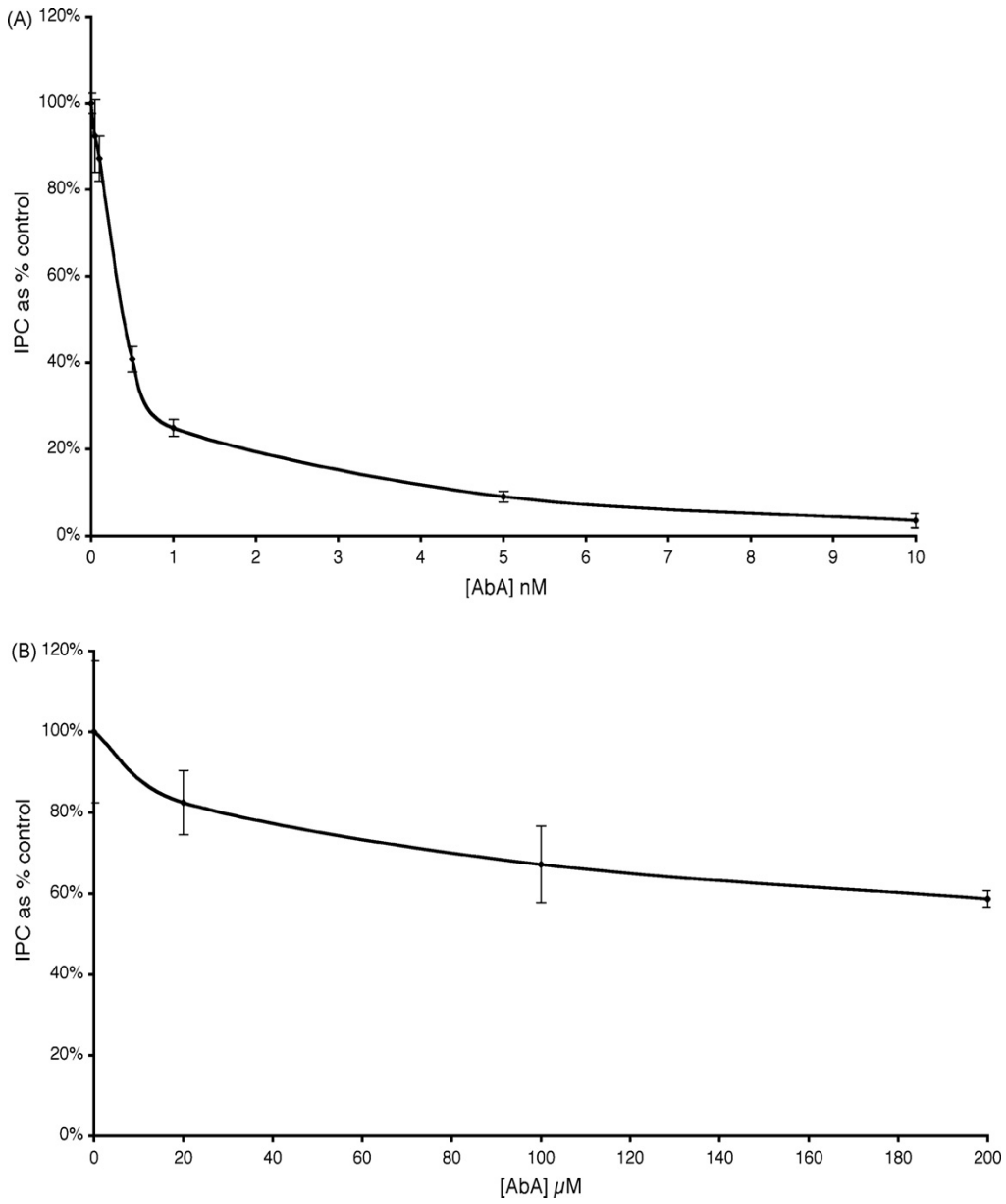

Fig. 5. The fungal IPC synthase inhibitor aureobasidin A is active against TbSLS activity. (A) TbSLS4 IPC synthase turnover determined using the described *in vitro* assay in the presence of aureobasidin A (AbA). The IC_{50} was calculated from the linear portion of the curve as being 0.42 nM. (B) *L. major* IPC synthase turnover was confirmed to be relatively refractory to AbA [10]. The IC_{50} was in excess of 100 μM, more than 200,000 times greater than that for TbSLS4. Turnover, as determined by IPC production, was scored as 100% in the absence of the inhibitor.

was completely inhibited and the parasites were scored as dead by light microscopy after 24 h. The EC_{50} of aureobasidin A against the parasites was estimated from these data as being below 250 nM. However, it is the trypanocidal activity of this compound at higher concentrations that is of most significance.

3.5. Summary

Previous studies using pharmacological and genetic inhibition of the first step in sphingolipid biosynthesis, catalyzed by serine palmitoyltransferase, showed that this pathway is essential for the viability of both bloodstream and procyclic forms of *T. brucei* [17,27]. This is in contrast to the related protozoan parasite *Leishmania major* where this enzyme, though essential for sphingolipid biosynthesis, is non-essential for both viability and pathogenesis [28,29]. These studies indicate that sphingolipid biosynthesis could be a viable drug target in the African trypanosomes.

The sphingolipid biosynthetic pathway is largely conserved across the Eukaryota. However, whilst animal cells synthesize the phosphosphingolipid SM, yeast and plants, plus at least some protozoa, produce IPC [7]. The IPC synthase of pathogenic fungi has long been validated and studied as a drug target [11], and the recent identification of a functional orthologue in the protozoan Kinetoplastids, the causative agents of several so-called neglected diseases, has led to its consideration as a target for anti-protozoal agents [10]. In this study we confirm that two of the four closely related *T. brucei* orthologues (TbSLS4 and 1) of the *Leishmania* IPC synthase (LmIPCS) are also functional orthologues of the *S. cerevisiae* enzyme encoded by *AUR1*. However surprisingly, unlike the *Leishmania* enzyme (and AUR1) TbSLS4 and 1 are able to catalyze the synthesis of both IPC and SM, which reflects the known sphingolipid content of *T. brucei* cells [15]. In an *in vitro* assay system utilizing TbSLS4 complemented *AUR1* mutant yeast microsomes, it was demonstrated that the *T. brucei* enzyme was able to function as an IPC synthase. Although PC

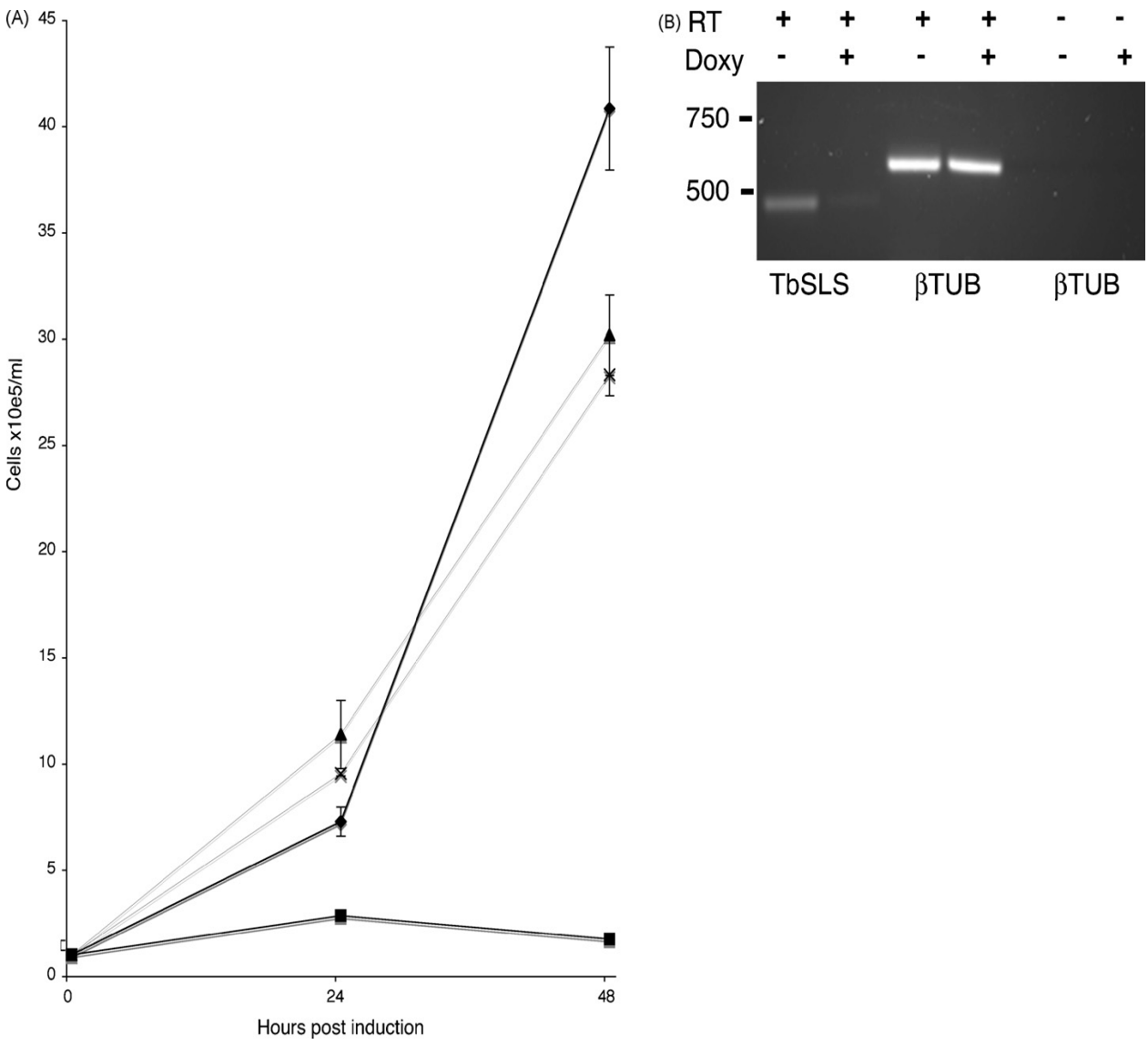

Fig. 6. TbSLS is an essential enzyme in bloodstream form *T. brucei*. (A) Inhibition RNA of TbSLS. Cell counts over a 48 h period: (▲) mock transfected cells non-induced; (×) mock tranfected cells induced by 1 μg/ml doxycline; (♦) TbSLS RNAi cells non-induced; (■) TbSLS RNAi cells induced by 1 μg/ml doxycline. Error bars for standard deviation over three replicates are shown. (B) RT PCR using total RNA isolated from TbSLS RNAi parasites with or without doxycycline induction (Doxy). RT, reverse transcriptase. βTUB, β tubulin control.

(and PE) was demonstrated to be a competitive binder with respect to PI, SM synthase activity could not be constituted possibly due to some level of specificity with respect to the donor substrate PC. Clearly, substrate specificity and the mechanism of action of TbSLS4 warrant further investigation to disentangle the detected IPC synthase function from the apparent SM synthase activity. Determination of the kinetic parameters of TbSLS4 would facilitate this, for example with respect to determining the binding constants for the apparent competitive IPC synthase inhibitors—PC and PE. In this respect it should be noted that the *T. brucei* enzyme is unlike its orthologues from the other kinetoplastid parasites where both the *Leishmania* (Fig. 5B) and *Trypanosoma cruzi* enzymes (Casbon and Denny, unpublished) demonstrate only IPC synthase activity in the *in vitro* system employed here. It is also clearly important to fully analyse the other three TbSLS isoforms to determine their function.

The known yeast and fungal IPC synthase inhibitor, aureobasidin A, has previously been shown to be active against the related kinetoplast, *Leishmania* species, inhibiting growth, but not affecting viability, in culture [30]. However, it has been demonstrated that the *L. major* IPC synthase is refractory to aureobasidin A (Fig. 5B) and that its affect against this species in culture is non-specific [28]. A similar situation has been observed with respect to the causative agent of Chagas disease, *T. cruzi* [19]. In contrast, this study showed that aureobasidin A demonstrated a high level of efficacy against TbSLS4 turnover *in vitro*, with an IC_{50} of 0.42 nM. In light of these results demonstrating the ability of a known inhibitor to affect enzyme activity, it was important to validate TbSLS as a potential target of anti-trypanosome drugs. Simultaneous RNAi of all four closely related isoforms of TbSLS demonstrated that this enzyme is essential for growth and so represents a new, much needed anti-protozoal target. Furthermore, aureobasidin A proved highly effective and trypanocidal against cultured bloodstream form *T. brucei*, with a sub-micromolar EC_{50}.

Together these data raise the possibility of the discovery of a new generation of lead inhibitors directed against TbSLS, ultimately leading to novel drugs for the treatment of human African trypanosomiasis.

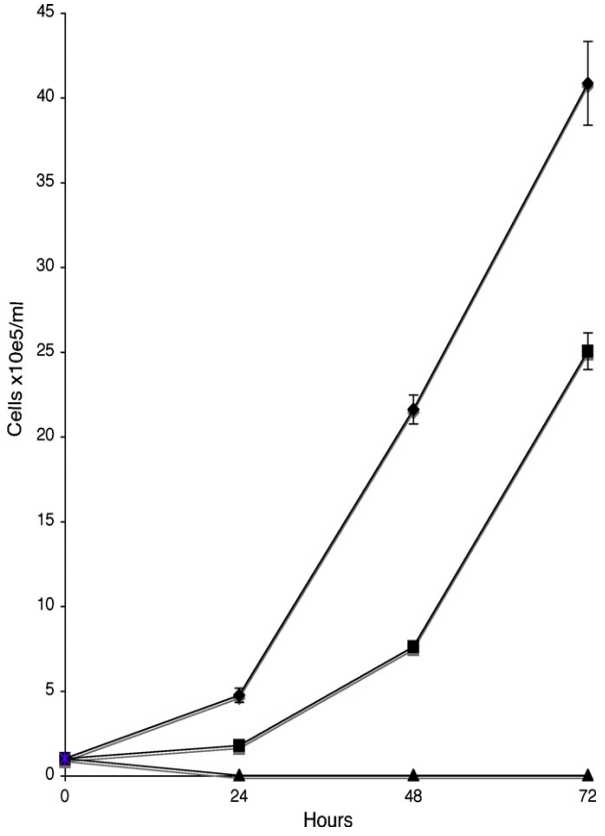

Fig. 7. Aureobasidin A is trypanocidal against bloodstream form *T. brucei*. Cell counts over 72 h with (▲); 1 μM aureobasidin A (AbA); (■) 250 nM AbA; (♦) control. Error bars for standard deviation over three replicates are shown.

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Functional analyses of differentially expressed isoforms of the Arabidopsis inositol phosphorylceramide synthase

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Abstract Sphingolipids are key components of eukaryotic plasma membranes that are involved in many functions, including the formation signal transduction complexes. In addition, these lipid species and their catabolites function as secondary signalling molecules in, amongst other processes, apoptosis. The biosynthetic pathway for the formation of sphingolipid is largely conserved. However, unlike mammalian cells, fungi, protozoa and plants synthesize inositol phosphorylceramide (IPC) as their primary phosphosphingolipid. This key step involves the transfer of the phosphorylinositol group from phosphatidylinositol (PI) to phytoceramide, a process catalysed by IPC synthase in plants and fungi. This enzyme activity is at least partly encoded by the *AUR1* gene in the fungi, and recently the distantly related functional orthologue of this gene has been identified in the model plant Arabidopsis. Here we functionally analysed all three predicted Arabidopsis IPC synthases, confirming them as aureobasidin A resistant AUR1p orthologues. Expression profiling revealed that the genes encoding these orthologues are differentially expressed in various tissue types isolated from Arabidopsis.

Keywords Arabidopsis · AUR1 · Expression · Inositol phosphorylceramide · IPC synthase · Sphingolipids

Abbreviations

AbA	Aureobasidin A
BSA	Bovine serum albumin
Cer	Ceramide
IPC	Inositol phosphorylceramide
NDB C_6-ceramide	6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoyl)sphingosine
PI	Phosphatidylinositol
SD	Synthetic minimal media with glucose
SGR	Synthetic minimal media with galactose
SM	Sphingomyelin

Introduction

The diverse, amphipathic sphingolipids consist of a long chain base backbone with long-chain fatty acid and polar

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alcohol attachments. These lipid species are ubiquitous membrane components of eukaryotic cells, as well as being found in some prokaryotic organisms and viruses (Smith and Merrill 2002). Studies in mammals and yeast have shown that they are important structural components of membranes and also serve as bioactive molecules involved in cell signalling and regulation (Dickson et al. 2006; Fernandis and Wenk 2007; Hanada et al. 1992). The unmodified sphingolipid, ceramide, is an intermediate in complex sphingolipid biosynthesis in the Golgi apparatus. These complex species ultimately concentrate in the outer leaflet of the plasma membrane where, with sterols, they form lipid raft microdomains (Futerman and Hannun 2004). Rafts have been proposed to be central to a multitude of cellular processes, from the polarized trafficking of lipid-modified proteins (Brown and London 1998) to the formation of signal transduction complexes (Magee et al. 2002; Pierce 2002). Furthermore, sphingolipid metabolites such as ceramide and phosphorylated sphingosine (sphingosine-1-phosphate), are central to intracellular signal transduction processes that regulate cell growth, differentiation and apoptosis (programmed cell death—PCD; Futerman and Hannun 2004).

The biosynthesis of sphingolipids shows commonality between mammals, fungi and plants up to the formation of ceramide (phytoceramide in plants and fungi), but the predominant complex phosphosphingolipid species subsequently synthesized differs. Mammals produce sphingomyelin (SM; a ceramide unit with a phosphorylcholine moiety), whereas fungi and plants synthesize inositol phosphorylceramide (IPC) by the transfer of the phosphorylinositol group from phosphatidylinositol (PI) to phytoceramide, a reaction catalysed by IPC synthase. This enzyme was shown to be at least partly encoded by the *AUR1* gene in yeast and other fungi (Nagiec et al. 1997), and recently an accessory protein involved in IPC synthase activity (KEI1p) in these organisms has been identified (Sato et al. 2009). However, the plant enzyme remained unidentified although the activity had been measured in bean microsomes (Bromley et al. 2003). Then, during a screen for factors involved in PCD defence mechanisms, an *AUR1* functional orthologue was identified in Arabidopsis. However, the encoded protein demonstrated little homology to the yeast AUR1p, having most similarity to the more recently identified animal sphingomyelin synthases (Wang et al. 2008). This lack of similarity to the long known fungal enzyme (Nagiec et al. 1997) lay behind the previously fruitless search for the plant orthologue (Dunn et al. 2004).

Here we demonstrate that all three orthologues identifiable in the Arabidopsis genome database represent aureobasidin A resistant functional orthologues and that, through phylogenetic analyses, these represent a new group of sphingolipid synthases within the wider enzyme family. Furthermore, we show the expression profile of all three isoforms in the tissues of Arabidopsis. This demonstrated that there is differential expression of IPC synthase, an enzyme central to the synthesis of plasma membrane sphingolipids (Dunn et al. 2004) and a regulator of PCD (Wang et al. 2008) in this plant species.

Materials and methods

Complementation of auxotrophic AUR1 mutant yeast with AtIPCS1-3

Arabidopsis IPC synthase 1, 2 and 3 (AtIPCS1–At3g54020.1, AtIPCS2–At2g37940.1 and AtIPCS3–At2g29525.1) were amplified from Arabidopsis cDNA using primers 5′ AtIPCS1 EcoRI gaattcATGACGCTTTATATTCGCCGCG and 3′ AtIPCS1 SalI gtcgacGAGCAGAGATCTCATGTG CC; 5′ AtIPCS2 EcoRI gaattcATGACACTTTATATTCG TCGTG and 3′ AtIPCS2 SalI gtcgacTCACGCGCCATT CATTGTG; 5′ AtIPCS3 EcoRI gaattcATGCCGGTTTAC GTTGATCGCG and 3′ AtIPCS3 SalI gtcgacTCAATGAT CATCTGCTACATTG.

The products were subsequently cloned into the yeast vector pRS426MET25, creating pRS426 AtIPCS1-3. In the YPH499–HIS–GAL–AUR1 *S. cerevisiae* strain expression of the essential *AUR1* gene (Nagiec et al. 1997) is under the control of the *GAL1* promoter and is repressed in the presence of glucose (Denny et al. 2006). YPH499–HIS–GAL–AUR1 was transformed with pRS426 AtIPCS1-3 and pRS426 AUR1 and functionally complemented transformants selected on non-permissive SD medium containing necessary nutritional supplements (Denny et al. 2006).

Diffusion assay of complemented auxotrophic AUR1 mutant yeast

YPH499–HIS–GAL–AUR1 pRS246 AtIPCS1-3 and YPH499–HIS–GAL–AUR1 pRS246 AUR1 were assayed for susceptibility to aureobasidin A (Takara) and myriocin (Sigma) as previously described (Nagiec et al. 2003). Briefly, 2.4×10^7 logarithmically dividing cells were embedded in 15 ml of YPD-agarose (1% yeast extract, 2% peptone, 2% dextrose, 0.8% agarose) on 100 cm^2 square Petri dishes (Sarstedt). Inhibitors were applied in Me_2SO at the concentrations described below and the dishes incubated at 30°C.

Microsomal assay of AtIPCS

For in vitro assay microsomal membranes were prepared from YPH499–HIS–GAL–AUR1 pRS246 AtIPCS1-3 and YPH499–HIS–GAL–AUR1 pRS246 AUR1 and assayed as

described (Mina et al. 2009). In brief, assays were performed in 50 µl of 100 mM Tris HCl pH 7.4, 10 mM EDTA and 6 mg/ml defatted BSA, with or without 1 mM PI (soybean, Avanti Polar Lipids; predominant species C16:0-C18:2) and 5 µM aureobsidin A, and with 2 µl of microsomes (10 mg/ml protein) and 2 µl of 5 mM NBD C_6-Ceramide (Molecular Probes) in DMSO. After incubation at 30°C for 60 min the reaction was stopped by the addition of 150 µl of chloroform/methanol/water (10:10:3 v/v/v) and the lipid fraction isolated by phase separation. This process facilitated reproducible quantitative analyses of IPC formation. Following equilibriation (Synergy HT, Bio-tek) samples were analysed by high performance thin-layer chromatography (Denny et al. 2001; Ralton and McConville 1998), imaged using a FLA3000 scanner (Fuji) and quantified using the Aida V3.11 software package.

Plant growth and harvesting of material

Arabidopsis thaliana (Col-0) plants were grown under long-day conditions (16 h day/8 h night) and the following tissues were harvested at specific development stages (Boyes et al. 2001). Rosette leaves were collected at stage 3.90, cauline leaves and stem were collected at stage 5.10, roots and flowers were collected at stage 6.50, siliques were collected at stage 8.00.

RNA isolation and RT

Plant tissue was harvested and immediately frozen in liquid nitrogen. Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen), including DNAse I digestion, according to the manufacturers instructions.

RNA integrity was tested by electrophoresis on 1% agarose gels and was quantified by measuring the absorbance at 260 nm. The $A_{260\ nm}/A_{280\ nm}$ ratios of purified RNA samples were in the range of 2.0 to 2.1 and were determined to be free of genomic DNA by the absence of larger, intron containing, PCR products.

RT reactions were carried out on 1.0 µg of total plant RNA using Superscript III reverse transcriptase (Invitrogen) and oligo dT primer according to the manufacturers instructions.

Real-time qRT-PCR conditions and analysis

Steady-state levels of RNA for each AtIPCS gene in plant material were analysed using SYBR green to monitor DNA synthesis with a Rotor-Gene RG-3000 (Corbett Research) instrument. Amplification was achieved with the following gene-specific primers; AtIPCS1-forward 5′-AGCCTCTTG ATTATTGCGTC-3′ and AtIPCS1-reverse 5′-AACAACG GCATTGCTCCCT-3′ to give a 145 bp product; AtIPCS2-forward 5′-AGCCTCTTGATCATTGCCTC-3′ and AtIPCS2-reverse 5′-GACTGCTGTGTTGCTCCCA-3′ to produce a 145 bp product; AtIPCS3-forward 5′-TGGCTTAT GGCAGTAATACAG-3′ and AtIPCS3-reverse 5′-GCCAG AAATGGCAGAACGTTCT-3′ to produce a 141 bp product. To determine the highest specificity and sensitivity qPCR profile for each AtIPCS transcript, titration experiments were performed over a range of $MgCl_2$ concentrations (1.5 to 5 mM) and primer concentrations from (0.1 to 0.5 µM). Concentrations were considered optimal at the lowest C_T value that reproducibly gave an amplicon of the correct size in the absence of primer dimers. Based on results of these optimization experiments, qPCR assays for the AtIPCS1 transcript contained 3 mM $MgCl_2$ and 0.5 µM of each primer; the AtIPCS2 transcript 3 mM $MgCl_2$ and 0.4 µM of each primer; and the AtIPCS3 transcript 4 mM $MgCl_2$, 0.4 µM of the forward and 0.5 µM of the reverse primer. The reactions (total volume 20 µl) also contained 2 µl of 10× PCR buffer, 10 pg of cDNA, 200 µM dNTPs, SYBR Green I dye and 2 Units of Taq polymerase. Assays were carried out using the following conditions: 1 cycle of 10 min of 95°C, followed by 30 cycles of 10 s of 95°C, 15 at 52°C and 20 s of 72°C. Amplicon dissociation curves were recorded at the end of the PCR cycles. External standard curves were constructed using the three AtIPCS cDNAs and relevant gene-specific primer pairs and used to determine the number of transcripts for each gene.

Results

AtIPCS1-3 complement an *AUR1* auxotrophic mutant yeast and confer aureobasidin A resistance

Interrogation of the Arabidopsis genome database with the predicted protein sequence of the protozoan IPC synthase from *Leishmania major* (*Lm*IPCS) (Denny et al. 2006) identified opening reading frames encoding three highly related sequence orthologues—Arabidopsis IPC synthase 1 (AtIPCS1–At3g54020.1), AtIPCS2 (At2g37940.1) and AtIPCS3 (At2g29525.1)—demonstrating 26, 29 and 31% identity to the protozoan protein. Subsequently, AtIPCS2 has been isolated, characterised and designated ERH1 (Wang et al. 2008). The open reading frames of AtIPCS1-3, when cloned into an URA3 selectable expression vector to create pRS426 AtIPCS1-3, restored the growth of the *AUR1* auxotrophic mutant YPH499-HIS-GAL-AUR1, as did the ectopic expression of *S. cerevisiae* AUR1p (AUR1) (Fig. 1a). These data indicated that AtIPCS1, 2 and 3 are functional orthologues of fungal AUR1p, a protein that forms at least part of an IPC synthase (Nagiec et al. 1997).

By diffusion assay (Nagiec et al. 1997, 2003) the efficacy of two different classes of inhibitor were assayed

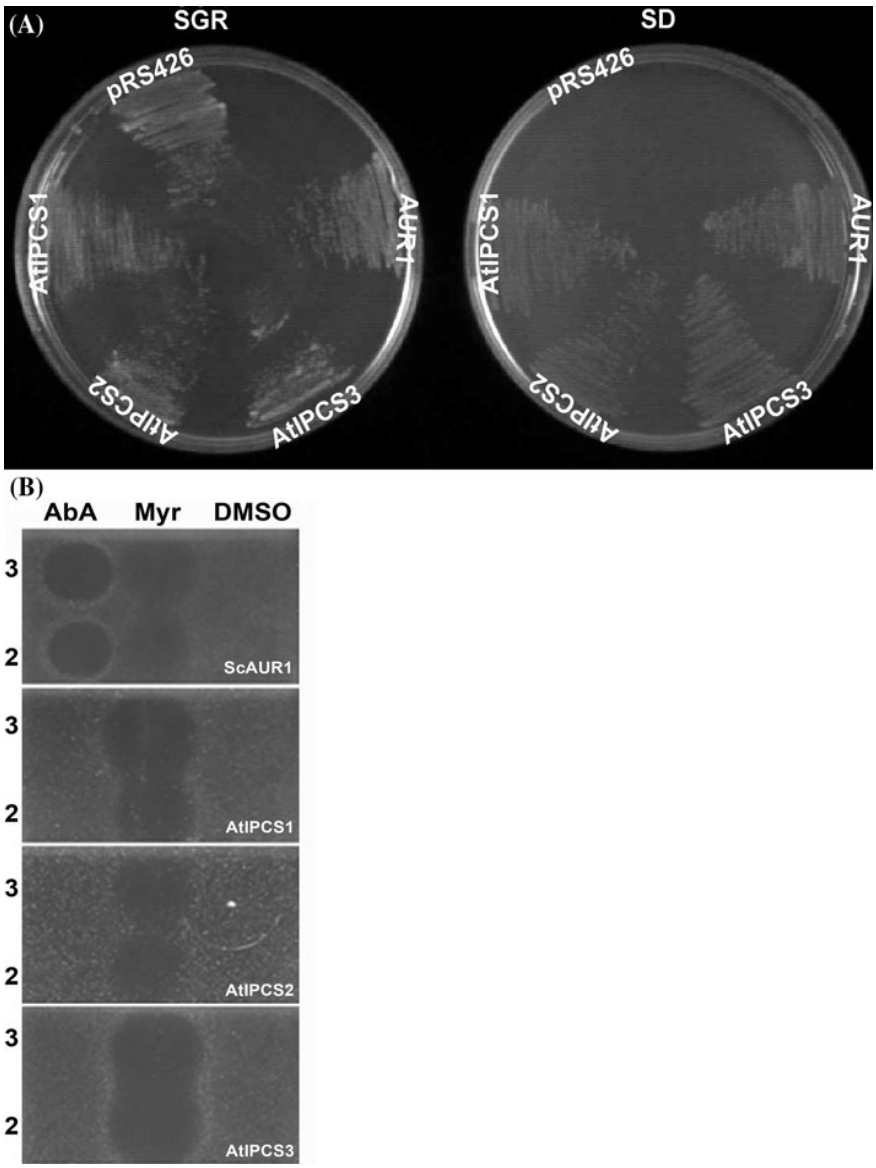

Fig. 1 AtIPCS1-3 complements a yeast *AUR1* auxotrophic mutant and confers aureobasidin A resistance. **a** YPH499-HIS-GAL-AUR1 auxotropic yeast transformed with pRS246MET25 either empty or bearing AUR1 or AtIPCS1-3 and grown under permissive (SGR) or non-permissive conditions (SD). **b** 100μM aureobasdin A (AbA) in DMSO, 1 mM myriocin (Myr) in DMSO and DMSO alone spotted in 2 and 3 μl quantities onto YPH499–HIS–GAL–AUR1 complemented with AUR1 or AtIPCS1-3 on non-permissive SD plates. pRS426—YPH499–HIS–GAL–AUR1 pRS426MET25; AUR–YPH499–HIS–GAL–AUR1 pRS426 ScAUR1; AtIPCS1-3–YPH499–HIS–GAL–AUR1 pRS426 AtIPCS1-3. Grown on either permissive (non-glucose containing) SGR -HIS -URA or non-permissive (glucose containing) SD–HIS–URA

against YPH499-HIS-GAL-AUR1 pRS426 AtIPCS1, 2 and 3. The transgenic yeast were resistant to aureobasidin A (AbA, a fungal IPC synthase inhibitor) at 100 μM (Fig. 1b), a concentration shown to inhibit the growth of *Lm*IPCS complemented yeast (Denny et al. 2006), but remained sensitive to myriocin (which inhibits the first step in sphingolipid biosynthesis—serine palmitoyltransferase, SPT). Indeed, yeast complemented with the plant enzymes appeared to be more sensitive to the SPT inhibitor than the AUR1 control, a similar observation had previously been made with regard to the *Lm*IPCS complemented yeast (Denny et al. 2006). The in vitro assay for AtIPCS1-3 described below (Fig. 2a) confirmed that the Arabidopsis enzymes are AbA insensitive. In contrast, the IPCS activity from Golden butterwax bean extracts has been shown to be acutely sensitive to this inhibitor (Bromley et al. 2003). The reasons for this difference in AbA sensitivity are unclear and no data are currently available, for the bean sequence, to help elucidate this discrepancy.

AtIPCS1-3 are functional IPC synthases

In vitro assay of microsomes prepared from YPH499-HIS-GAL-AUR1 pRS426 AtIPCS1-3 demonstrated that the three plant enzymes are active IPC synthases (Fig. 2a). However, their turnover appeared low when compared to an *S. cerevisiae* AUR1 control. This was also noted by Wang et al. (2008) and was unaffected by the use of NBD C_6-phytoceramide rather than C_6-ceramide as acceptor substrate. In support of the data shown above (Fig. 1b), AtIPCS1-3 activity was insensitive to the addition of AbA at a concentration (5 μM) that completely inhibited the

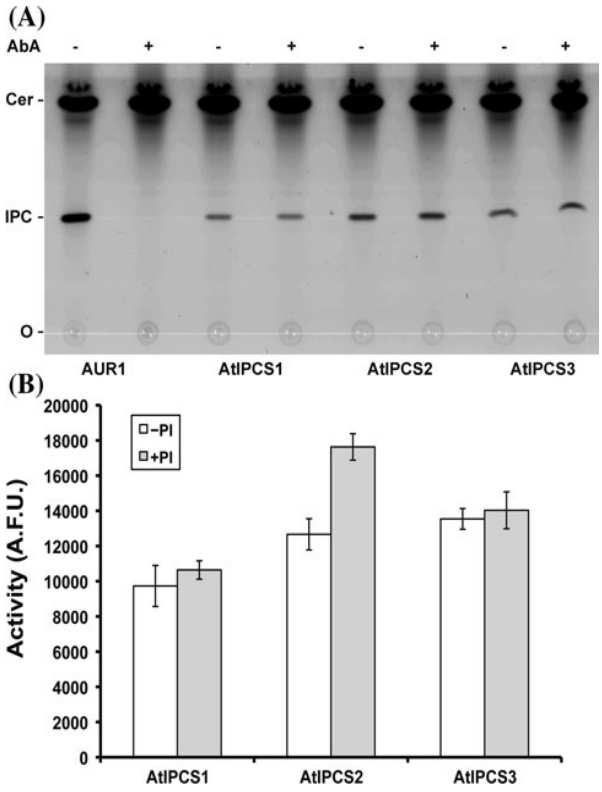

Fig. 2 AtIPCS1-3 encode functional IPC synthases. In vitro assay of microsomal extracts from YPH499-HIS-GAL-AUR1 pRS246 AtIPCS1-3 and YPH499-HIS-GAL-AUR1 pRS246 AUR1. Assay in the presence of phosphatidylinositol (PI) with (+) or without (−) the specific fungal inhibitor aureobasidin A (AbA). In contrast to the *S. cerevisiae* IPC synthase (AUR1), the plant activity encoded by AtIPCS1-3 is insensitive to AbA at 5 μM. Markers of NDB C_6-ceramide and NBD C_6-IPC, and the origin (O) indicated. Assay with (+) or without (−) donor substrate PI in the presence of 5 μM AbA. Enzyme turnover is only significantly enhanced by the addition PI in the case of AtIPCS2. Mean of 3 separate experiments, standard error shown. A.F.U.—Arbitrary Fluorescence Units

yeast enzyme (Fig. 2a). Thereafter this inhibitor was applied to the reaction mix for assay of AtIPCS1-3 at 5 μM.

In the experiment (Fig. 2a) above the plant (and yeast) enzymes are assayed in the presence of exogenous soybean PI. To ascertain the effect of adding this exogenous donor substrate samples were assayed with and without PI in the reaction mix (Fig. 2b). All samples showed a similar level of activity in the absence of donor substrate but, surprisingly, only AtIPCS2 demonstrated any significant increase in turnover on the addition of PI. This indicated that the AtIPCS1 and 3 are unable to utilize substrate from this source effectively. Similar results were obtained utilizing bovine PI which has previously been demonstrated to be effectively utilized by the protozoan orthologues from both *L. major* and *Trypanosoma brucei* but not by *S. cerevisiae* AUR1p (Mina et al. 2009). Despite this, these data confirm AtIPCS1-3 as functional orthologues of AUR1p, forming at least part of an IPC synthase.

The plant IPC synthases form a new class of sphingolipid synthase

The key motifs, D3 and D4, of the sphingolipid synthase family are conserved in the plant IPC synthases (Wang et al. 2008). However, unlike their protozoan and mammalian counterparts they lack a D1 domain (Huitema et al. 2004). In this they resemble the fungal IPC synthases and it may be hypothesized that D1 is involved in the binding of ceramide, a substrate for the protozoan and mammalian enzymes, whereas another unidentified region is involved in binding the phytoceramide substrate to the plant and fungal IPC synthases. Despite the conservation of some of these motifs the plant sequences are divergent with respect to primary sequence and so, until recently, remained cryptic within the genome databases (Wang et al. 2008). This divergence was illustrated by phylogenetic analysis of predicted SM and IPC synthase sequences which demonstrated that the plant enzymes, the three orthologues identified in the *Oryza sativa* (rice) database are included, form a new clade of sphingolipid synthases (Fig. 3).

Expression profiling of AtIPCS1-3

Estimates of the relative abundance of transcripts can be made from their representation within the EST databases. For the three AtIPCS genes described a similar number of AtIPCS1 and AtIPCS3 transcripts are present, whilst the largest number is for AtIPCS2 (Table 1; data derived from TAIR BLAST version 2.2.8). However, these data do not give any information regarding the tissue location of the transcripts and are therefore of limited use when investigating the expression of gene families. Tissue-specific transcript abundance data can be obtained from multiple microarray experiments using Genevestigator (Grennan 2006). Only AtIPCS2 is represented on the Affymetrix arrays that are used to derive these data and this shows that the gene is expressed in cauline leaves, roots and rosette leaves at similar levels and these are approximately twice the expression levels seen in stems, flowers and siliques (Table 2).

Given the limitations of these data sets, in order to establish a complete expression profile for each AtIPCS isoform, real time quantitative RT-PCR was performed on RNA isolated from various Arabidopsis tissues (Fig. 4). AtIPCS2 was the most highly expressed of the three isoforms in all tissues. In agreement with the Genevestigator data, the highest levels of AtIPCS2 transcript were seen in root, rosette leaves and cauline leaves and these were five to ten fold greater than the levels of transcripts in siliques,

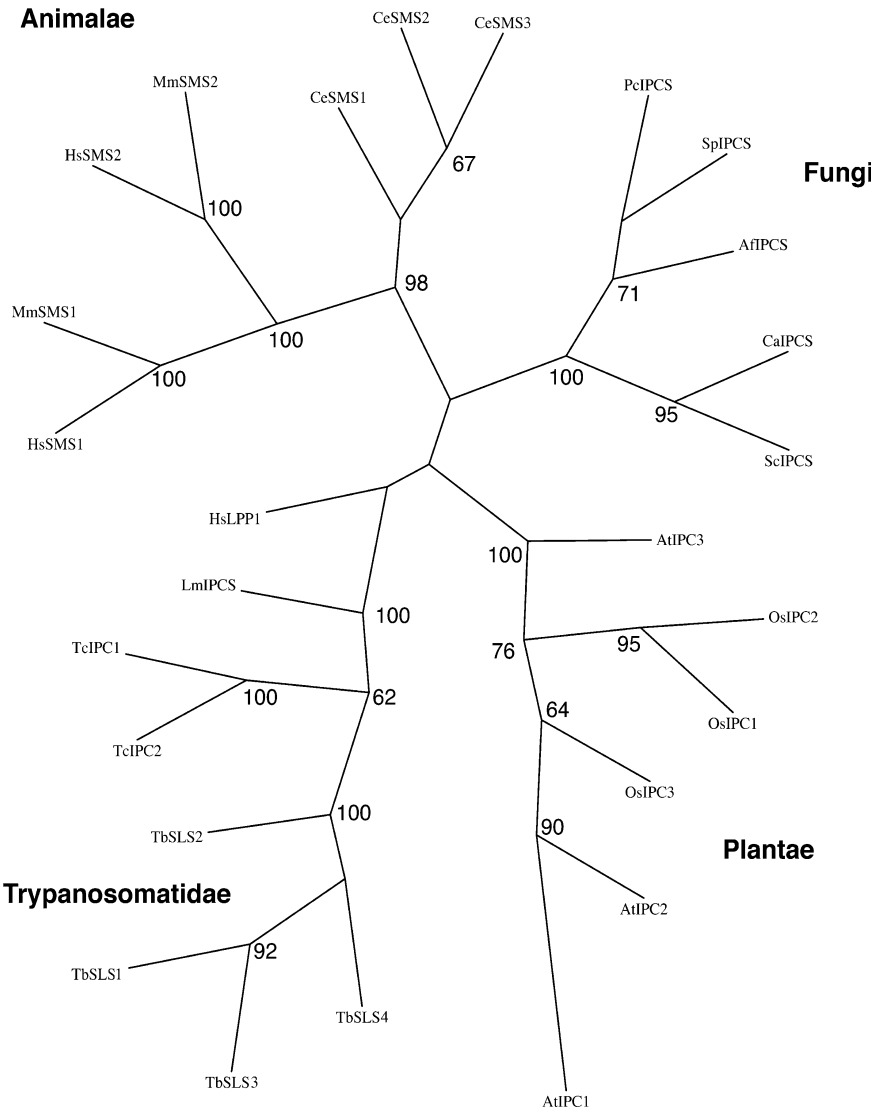

Fig. 3 The plant IPC synthase defines a new class of sphingolipid synthases. Maximum parasimony analysis of Animalae, Fungi, Trypanosomatidae and Plantae sphingolipid synthase sequences. Bootstrap scores >60 indicated. *Homo sapiens* LPP1 (outgroup) accession number: O14494; *Arabidopsis thaliana* IPCS1-3 accession numbers: At3g54020.1, At2g37940.1, At2g29525.1; *Oryza sativa* IPCS1-3: NP_001044812, NP_001055712, NP_001055096; *T. brucei* SLS1-4: Tb09.211.1030, Tb09.211.1020, Tb09.211.1010, Tb09.211.1000; *T. cruzi* IPCS1&2: Tc00.1047053506885.124, Tc00.1047053510729.290; *L. major* IPCS: LmjF35.4990; *Aspergillus fumigatus* AUR1p: AAD22750; *Candida albicans* AUR1p: AAB67233; *Pneumocystis carinii* AUR1p: CAH17867; *Saccharomyces cerevisiae* AUR1p: NP_012922; *Schizosaccharomyces pombe* AUR1p: Q10142; *Caenorhabditis elegans* SMS1-3: Q9U3D4, AAA82341, AAK84597; *Homo sapiens* SMS1&2: AB154421, Q8NHU3; *Mus musculus* SMS1&2: Q8VCQ6, Q9D4B1

Table 1 EST numbers for AtIPCS genes derived from TAIR BLAST version 2.2.8

	EST
AtIPCS1	32
AtIPCS2	57
AtIPCS3	25

Table 2 Microarray data for AtIPCS2 in the genevestigator database

Root	Rosette leaf	Cauline leaf	Stem	Flower	Siliques
2383	2135	2509	1167	998	705

stems and flowers. The patterns of expression determined by these two methods are in general agreement but the relative levels differ, this may reflect differences in the plant material used in the each case. Genevestigator uses a large number of microarray experiments to generate data and is therefore robust, however the data represents plant material grown under different conditions and which may have been harvested at different times within a developmental stage. The quantitative RT-PCR data presented here is determined from plants grown and harvested under identical conditions and therefore represents transcript levels at defined developmental stages in these tissues.

AtIPCS1 transcripts are expressed at low levels in all tissues, ranging from 0.05% of AtIPCS2 levels in rosette leaves to 2.8% of AtIPCS2 levels in stems. While AtIPCS3 transcripts are also present in low levels in cauline leaves, rosette leaves, roots and stems (ranging from 0.02 to 0.67% of AtIPCS2 transcripts) they are present at similar levels to AtIPCS2 transcripts in stem (84% of AtIPCS2 levels) and flower (74% of AtIPCS2 levels).

The data presented here adds to existing studies by providing tissue specific expression data for all the

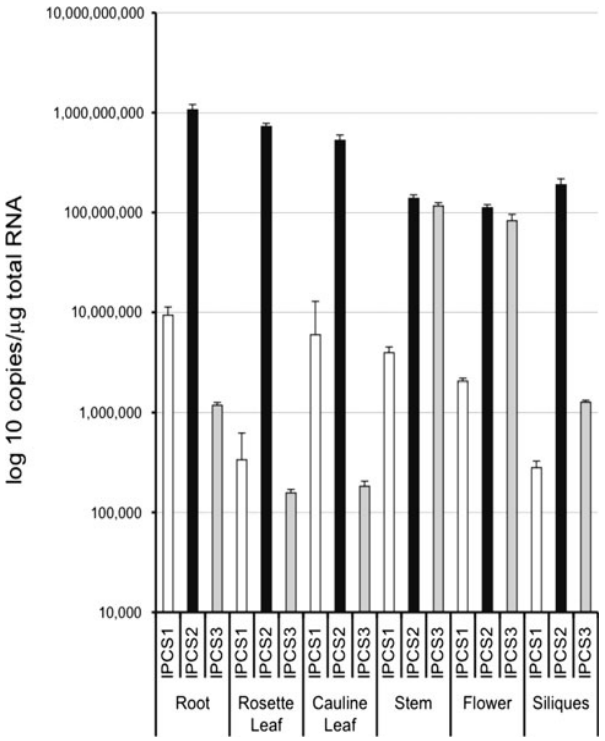

Fig. 4 Steady-state levels of AtIPCS isoform mRNAs in plant tissues. The numbers of AtIPCS1-3 transcripts were determined in total RNA from various tissues, by quantitative RT-PCR using a standard curve for each transcript. AtIPCS2 (At2g37940.1) is the most abundant form in roots, leaves and siliques, whereas stems and flowers have similar numbers of AtIPCS2 and AtIPCS3 (At2g29525.1) transcripts. AtIPCS1 (At3g54020.1) transcript is uniformly low in all tissues

isoforms of AtIPCS which may indicate a specific role for AtIPCS3 in stems and flowers.

Discussion

The data presented describe the analyses of all three orthologues of the recently identified Arabidopsis IPC synthase (Wang et al. 2008). They all represented functional orthologues of *S. cerevisiae* IPC synthase, AUR1p, and demonstrated activity in an in vitro assay. However, unlike the yeast activity the Arabidopsis enzyme is resistant to aureobasidin A, a non-competive inhibitor of the fungal IPC synthase with an unknown mechanism of action (Zhong et al. 1999). Conversely, it has previously been shown that the IPCS activity in Golden butterwax bean extracts was acutely sensitive to this inhibitor (Bromley et al. 2003). The reasons for this discrepancy are unclear, however it is notable that similarly profound differences in aureobasidin A sensitivity have been observed within the kinetoplastid parasite IPCS orthologues. Whilst the *L. major* enzyme is relatively refractory to the drug, the *T. brucei* sphingolipid synthase (TbSLS) is highly sensitive (Mina et al. 2009). No reason for this can be deduced from the primary sequence data and it may be envisaged that this diversity is due to subtle differences in 3-dimensional enzyme structure.

In the in vitro, microsome-based assay all three Arabidopsis isoforms demonstrated IPC synthase turnover. However, although the presence of PI (from soybean or bovine sources) led to a significant increase in turnover with AtIPCS2, AtIPCS1 and 3 were refractory to the addition of this donor substrate. This indicated that PI from these sources was utilized inefficiently by these Arabidopsis isoforms. Similarly, it has been noted that mammalian PI is not efficiently utilized by the *S. cerevisiae* IPC synthase activity (Mina et al. 2009). Notably, unlike the yeast phospholipids (Guan and Wenk 2006), plant and mammalian PIs exhibit varying degrees of polyunsaturation (Thompson and MacDonald 1975, 1976). It may be hypothesized this polyunstauration may confer structural constraints and make it less acceptable to the *S. cerevisiae* enzyme. However, the lack of reactivity of AtIPCS1 and with plant derived PI (soybean, C16:0–C18:2) is less easy to explain. It is possible that the endogenous yeast PI has a higher affinity for these isoforms and outcompetes the exogenous substrate. Attempts to minimize the available quantity of endogenous PI to test this were made by detergent washing as previously (Mina et al. 2009). However, this led to apparent inactivation of the enzymes. Alternatively, significant differences may exist in the substrate requirements of these Arabidopsis isoforms. Notably, AtIPCS1 transcript level is low in all plant tissue and AtIPCS3 in many, including siliques, perhaps reflecting the level of acceptable donor PI in seeds (such as soybeans, the source of PI in these experiments).

Despite these variances, all the plant IPC synthase orthologues identified share the motifs predicted to form the catalytic triad that promotes nucleophilic attack on lipid phosphate ester bonds which, in the case of IPC synthase, is thought to lead to the transfer of an inositol phosphate group from PI to the 1-hydroxyl group of (phyto)ceramide releasing diacylglycerol as a by-product (Huitema et al. 2004; Neuwald 1997). Therefore it is likely that the eukaryotic sphingolipid synthases possess a common mechanism of action, indicating a single evolutionary origin. In contrast, the prokaryotic SM synthase lacks the catalytic triad (Luberto et al. 2003) and is likely to have evolved independently. Phylogenetic analyses show the plant proteins forming a distinct clade, thereby defining a new class of eukaryotic sphingolipid synthases.

It has been demonstrated, by RNAi of the first enzyme in the biosynthetic pathway—serine palmitoyltransferase, that sphingolipid biosynthesis per se is essential for the viability of Arabidopsis (Chen et al. 2006). Furthermore,

recent work has suggested that the plant IPC synthase is pivotal in pathogen-resistance associated PCD. By analyses of leaf tissue from an ERH1 (AtIPCS2) insertion mutant Wang et al. (2008) detected a significant increase in ceramide levels, an observation consistent with the fact that ceramide is a substrate for IPCS. Importantly, ceramide is a key regulator of PCD (Futerman and Hannun 2004) and the accumulation of this factor was co-incident with PCD at the site of powdery mildew infection in Arabidopsis (Wang et al. 2008). This work is further supported by similar observations in accelerated-cell-death (*acd11* and *acd5*) mutants of Arabidopsis in which a sphingosine transfer protein (ACD11) or a ceramide kinase (ACD5) are affected (Brodersen et al. 2002; Liang et al. 2003). Given the prominence of AtIPCS2 as a putative regular of PCD the differential expression levels of each of the three functional Arabidopsis orthologues may shed light on this key process in the whole plant.

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