Microbiology (2009), **155**, 1386–1396 **DOI** 10.1099/mic.0.025718-0

Review

Strategies for acquiring the phospholipid metabolite inositol in pathogenic bacteria, fungi and protozoa: making it and taking it

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myo-Inositol (inositol) is an essential nutrient that is used for building phosphatidylinositol and its derivatives in eukaryotes and even in some eubacteria such as the mycobacteria. As a consequence, fungal, protozoan and mycobacterial pathogens must be able to acquire inositol in order to proliferate and cause infection in their hosts. There are two primary mechanisms for acquiring inositol. One is to synthesize inositol from glucose 6-phosphate using two sequentially acting enzymes: inositol-3-phosphate synthase (Ino1p) converts glucose 6-phosphate to inositol 3-phosphate, and then inositol monophosphatase (IMPase) dephosphorylates inositol 3-phosphate to generate inositol. The other mechanism is to import inositol from the environment via inositol transporters. Inositol is readily abundant in the bloodstream of mammalian hosts, providing a source from which many pathogens could potentially import inositol. However, despite this abundance of inositol in the host, some pathogens such as the bacterium *Mycobacterium tuberculosis* and the protist parasite *Trypanosoma brucei* must be able to make inositol *de novo* in order to cause disease (*M. tuberculosis*) or even grow (*T. brucei*). Other pathogens such as the fungus *Candida albicans* are equally adept at causing disease by importing inositol or by making it *de novo*. The role of inositol acquisition in the biology and pathogenesis of the parasite *Leishmania* and the fungus *Cryptococcus* are being explored as well. The specific strategies used by these pathogens to acquire inositol while in the host are discussed in relation to each pathogen's unique metabolic requirements.

Introduction

In order to cause an infection, microbes must be able to live and proliferate inside the host. This necessitates the acquisition of essential nutrients by the pathogen. The pathogen must either obtain these nutrients from the host or synthesize the nutrients *de novo* from more basic compounds. The biosynthesis of phospholipids is an area that has received considerable attention in terms both of the insight it may give us regarding host–pathogen interactions and the possibility that an understanding of phospholipid metabolism may help in the identification of new drug targets (Vial *et al.*, 2003). Phospholipids are complex molecules that are essential for membrane integrity and intracellular signalling. The biosynthesis of these compounds requires a complex ordered set of biochemical steps involving a number of enzymes (Greenberg & Lopes, 1996; van Meer *et al.*, 2008; Vance, 2003; Vial *et al.*, 2003). An area of particular interest is understanding how pathogens acquire basic molecules such as *myo*-inositol, serine, choline or ethanolamine for the synthesis of phospholipids (Vial *et al.*, 2003). This review will focus on mechanisms used by diverse pathogens to obtain *myo*-inositol for the synthesis of the phospholipid phosphatidylinositol (PI).

In the last several years a number of studies have been performed to elucidate the roles that proteins involved in *myo*-inositol acquisition play in controlling virulence and/or viability in a variety of different pathogens (Chen *et al.*, 2008; Martin & Smith, 2005, 2006a; Movahedzadeh *et al.*, 2004). This review will cover the fascinating repertoire of mechanisms used by these different pathogens to acquire *myo*-inositol.

Inositol

myo-Inositol is a precursor for making PI, which is essential in all eukaryotes, including pathogenic fungi and protozoa, as well as in a small, but very significant group of eubacterial pathogens that includes the mycobacteria (Michell, 2008). In order to synthesize PI the pathogen must obtain *myo*-inositol. There are two main mechanisms for acquiring this molecule: synthesizing it *de novo* from glucose 6-phosphate or importing it from the host (Majumder *et al.*, 1997; Michell, 2008; Drew *et al.*, 1995; Einicker-Lamas *et al.*, 2000, 2007; Jin & Seyfang, 2003).

myo-Inositol is a polyol that is characterized as a six-carbon ring where each carbon is hydroxylated. A number of

isomers are biologically active, but *myo*-inositol is the most common (Majumder *et al.*, 1997; Michell, 2008). For the purposes of this review, *myo*-inositol will be referred to simply as inositol.

In eukaryotes inositol is used to make PI, which serves as a structural component of the membrane, but also as a precursor for several other very important lipid molecules including sphingolipids, ceramides and glycosylphosphatidylinositol (GPI) anchors (Michell, 2008). GPI anchors are used to attach a number of proteins to the plasma membrane and are essential in eukaryotic microbes (Ferguson *et al.*, 1994; Orlean & Menon, 2007). Certain GPI-anchored proteins play a special role in the virulence of both fungal and protozoan pathogens, serving as adhesins and/or variable epitopes to evade the immune system (Ferguson *et al.*, 1994; Sundstrom, 2002). PI is also the precursor for a wide variety of membrane-bound and non-membrane-bound phosphorylated inositol signal-transduction molecules (Michell, 2008; Strahl & Thorner, 2007). In addition, inositol serves as a 'compatible osmolyte' in some metazoan cell-types such as kidney and brain cells (Burg, 1997; Fisher *et al.*, 2002).

Mycobacteria are one of the few groups of eubacteria where PI is found in the membrane. PI serves as a precursor for the generation of more complex glycolipids that compose the outer cell wall of mycobacteria (Michell, 2008; Nigou *et al.*, 2003). In addition, the mycobacteria, along with other actinomycetes, use inositol to generate a thiol compound called mycothiol that serves many of the same functions as glutathione in other organisms, including detoxification and protecting the cell from oxidative damage (reviewed by Newton & Fahey, 2002).

Most eubacteria besides the *Actinomycetes* do not utilize inositol in their lipid membranes or as a redox molecule. However, a number of bacteria such as *Bacillus subtilis* (Yoshida *et al.*, 1997, 2008), *Aerobacter aerogenes* (Berman & Magasanik, 1966), *Rhizobium leguminosarum* bv. *viciae* (Fry *et al.*, 2001), *Sinorhizobium meliloti* (Galbraith *et al.*, 1998), *Corynebacterium glutamicum* (Krings *et al.*, 2006) and *Lactobacillus casei* (Yebra *et al.*, 2007) can utilize inositol as a carbon source. Even some microbial eukaryotes (such as *Cryptococcus* species) have been shown to use inositol as a carbon source (Healy *et al.*, 1977).

De novo biosynthesis of inositol

The *de novo* generation of inositol occurs by a universal mechanism that is conserved from eubacteria to archaea to eukaryotes (Majumder *et al.*, 1997). It depends on the tandem action of two enzymes. The first enzyme is inositol-3-phosphate synthase, which was first cloned from *Saccharomyces cerevisae* and was called Ino1p (Donahue & Henry, 1981), a name which will be used throughout this review. This enzyme, which converts glucose 6-phosphate to inositol 3-phosphate via a cyclization reaction, constitutes the rate-limiting step in *de novo* synthesis. NAD^+ serves as both a hydrogen acceptor and donor so no net NADH is formed in this reaction (Majumder *et al.*, 1997). The second enzyme in inositol synthesis is an inositol monophosphatase (IMPase), which dephosphorylates inositol 3-phosphate to create inositol (Michell, 2008). In *S. cerevisiae*, two redundant phosphatases, ScInm1p and ScInm2p, serve this purpose (Lopez *et al.*, 1999). The inositol synthesized by the sequential action of these enzymes can then be used to generate inositol-containing compounds such as PI or mycothiol that, in turn, can be used to make more complex molecules, depending on the organism.

Imported inositol

The alternative mechanism for obtaining inositol is to import it from the environment via inositol transporters. The two main types of transporters found in both metazoa and microbes are Na^+- or H^+-linked cotransporters, which both rely on ion gradients to transport inositol into cells against concentration gradients. In humans, *SMIT1* and *SMIT2* encode Na^+/inositol symporters (Coady *et al.*, 2002; Kwon *et al.*, 1992), and *HMIT* encodes a H^+/inositol symporter (Uldry *et al.*, 2001). A few inositol transporters have been characterized in human pathogens. For example, *Trypanosoma cruzi* has at least two different transporters based on a biochemical analysis of import activities, although neither transporter has actually been cloned. One transporter activity is Na^+-linked and the other is not (Einicker-Lamas *et al.*, 2000, 2007). In contrast to the case in *T. cruzi*, *Leishmania donovani* and *Candida albicans* have only one inositol transporter each, and in both cases the transporter is a H^+-linked symporter (Drew *et al.*, 1995; Jin & Seyfang, 2003). Inositol transporter activity has also been identified in the bacterial pathogen *Mycobacterium tuberculosis* as well as the environmental species *Mycobacterium smegmatis*. Although putative transporter genes have been identified bioinformatically in both *M. tuberculosis* and *M. smegmatis*, these genes have not yet been experimentally linked to transport activities by gene disruptions or transport assays (Movahedzadeh *et al.*, 2004; Newton *et al.*, 2006).

Mycobacterium species

In the pathogenic bacterium *Mycobacterium tuberculosis*, disruption of the inositol phosphate synthase gene *MtINO1* ablated the ability of the pathogen to cause disease in a mouse model of infection (Movahedzadeh *et al.*, 2004). The ability of *M. tuberculosis* to grow within macrophages is essential to virulence. Experiments done in tissue culture macrophages revealed that the *Mtino1*Δ mutant was killed more efficiently by macrophages than the wild-type strain (Movahedzadeh *et al.*, 2004). Thus, these results indicate that *M. tuberculosis* needs to generate inositol *de novo* in order to survive in a macrophage and cause disease, and cannot compensate by importing inositol from the host. This is not because *M. tuberculosis* lacks the ability to

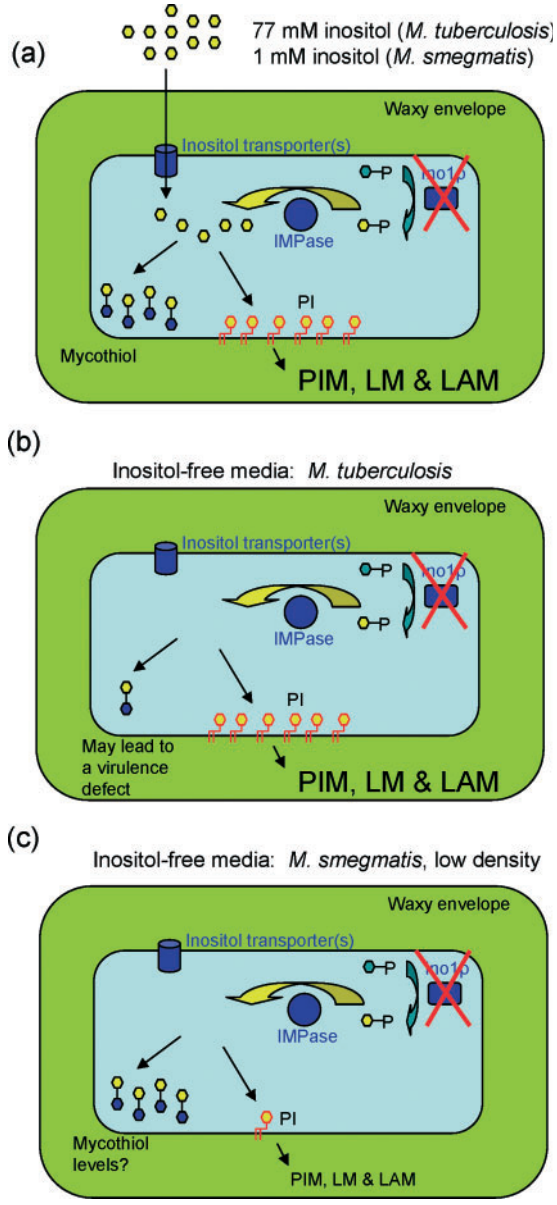

Fig. 1. Loss of the *MtINO1* gene in *Mycobacterium tuberculosis* causes a loss of viability and mycothiol in inositol-free media. It is unclear if the *Mtino1*Δ mutant also experiences a loss of lipoglycans under some growth conditions as has been observed for the *Mycobacterium smegmatis Msino1*Δ mutant. (a) The *Mtino1*Δ mutant can only grow like the wild-type strain in medium that contains at least 77 mM inositol, whereas the *Msino1*Δ mutant only requires 1 mM inositol in the medium for wild-type growth. (b) When the *Mtino1*Δ mutant is incubated in inositol-free medium there is a decrease in mycothiol levels, but not lipoglycans (PIM, LM and LAM). The *Mtino1*Δ mutant also does not lose viability under these conditions. Similar results were reported for the *Msino1*Δ mutant when incubated in inositol-free medium at high density, except that mycothiol was not measured. (c) When the *M. smegmatis Msino1*Δ mutant is incubated in inositol-free medium at a low dilution (i.e. exponential phase) it experiences a drop in lipoglycan levels and viability. Mycothiol levels were not measured. Yellow hexagon, inositol; green hexagon-P, glucose 6-phosphate; yellow hexagon-P; inositol 3-phosphate; red and yellow phospholipid, PI. These symbols also apply to Figs 2 and 3.

import inositol altogether. It may be because its ability to import inositol is not efficient. The *Mtino1*Δ mutant can only grow at wild-type rates on medium containing high inositol concentrations (⩾77 mM, Fig. 1a). It grows poorly in medium containing 10 mM inositol and not at all in medium with 1 mM inositol (Movahedzadeh *et al.*, 2004). The concentration of inositol in human serum is 61.0±12.4 μM and in rats it is 20–100 μM (Isaacks *et al.*, 1997; Kouzuma *et al.*, 2001; Palmano *et al.*, 1977), which suggests that there is an insufficient concentration of inositol to support growth of the *Mtino1*Δ mutant while it is in the host.

The 77 mM inositol concentration required to support growth of the *Mtino1*Δ mutant is very high when compared to other organisms. For example, an *S. cerevisiae Scino1*Δ mutant can grow on 10 μM inositol (Graves & Henry, 2000), and an *Msino1*Δ mutant of the environmental mycobacterium *M. smegmatis* can grow on 1 mM inositol (Fig. 1a) (Alderwick *et al.*, 2007; Haites *et al.*, 2005). The fact that the *Mtino1*Δ mutant requires such high concentrations of inositol suggests that it does not carry a true inositol transporter, but rather a sugar transporter that can also transport inositol at low efficiency. This question can be addressed when this transporter is cloned and characterized.

The reasons why the *Mtino1*Δ mutant is not virulent in mice and loses viability in macrophages are not clear. This is in part because there are conflicting data on which downstream inositol-containing molecules are affected by inositol depletion and what role these molecules have in virulence. There are two main types of molecules synthesized from inositol that affect growth and/or virulence characteristics. One molecule is PI, which is used to make inositol-containing lipoglycans such as phosphatidylinositol mannoside (PIM), lipomannan (LM) and lipoarabinomannan (LAM) that localize to the waxy envelope. The other molecule is mycothiol, which is used for redox chemistry (Alderwick *et al.*, 2007; Nigou *et al.*, 2003; Rawat & Av-Gay, 2007).

PI and PIM are required for viability, and LAM modulates the immune response in a manner required for virulence. LAM blocks maturation of the phagosome, which is important for *M. tuberculosis* to survive in the macrophage (reviewed by Briken *et al.*, 2004).

It is clear that PI, PIM and LAM are required for virulence; however, it is not clear if inositol depletion in the *Mtino1*Δ mutant causes a deficiency in their production. Analysis of the cell envelope of the *Mtino1*Δ mutant after incubation in inositol-free medium revealed that the mutant did not

experience a drop in these lipoglycans (Fig. 1b) (Movahedzadeh *et al.*, 2004). The mutant also did not suffer a loss of viability. Similar results were observed for an *ino1*Δ mutant made in the environmental mycobacterium *M. smegmatis* as long as it was not actively growing. The *Msino1*Δ mutant did not exhibit a decrease in viability or lipoglycan levels when incubated in inositol-free media at a high density, where it was not replicating (i.e. stationary phase). However, an opposite result was observed when the *Msino1*Δ mutant was diluted to a low density (i.e. exponential phase) in inositol-free medium. In exponential phase the *Msino1*Δ mutant lost viability over time, and the levels of cell wall lipoglycans decreased (Fig. 1c) (Haites *et al.*, 2005). It is not clear if the *Mtino1*Δ mutant will lose viability if it is incubated in inositol-free medium at a lower density (Movahedzadeh *et al.*, 2004). If an *Mtino1*Δ mutant loses viability and lipoglycan production during exponential growth, as seen in the *Msino1*Δ mutant, it may experience a decrease in lipoglycans during infection, which may contribute to its loss of viability and virulence.

Alternatively, it is also possible that *ino1*Δ mutants constructed in *M. smegmatis* and *M. tuberculosis* exhibit fundamental differences in how they respond to inositol deprivation. The *Mtino1*Δ mutant may simply arrest growth in inositol-free media, but remain viable, regardless of the concentration to which it is diluted. *M. smegmatis* and *M. tuberculosis* differ in a number of substantial ways. While *M. tuberculosis* is an intracellular parasite, *M. smegmatis* is an environmental saprophyte. In addition, *M. smegmatis* grows much faster than *M. tuberculosis* (doubling time of 4 h for *M. smegmatis* as opposed to 15–22 h for *M. tuberculosis*: Bhatt *et al.*, 1998; James *et al.*, 2000). In addition, the *Msino1*Δ mutant grows as well as wild-type in much lower concentrations of inositol (1 mM inositol for *Msino1*Δ as opposed to 77 mM for *Mtino1*Δ: Haites *et al.*, 2005). The effect that the loss of *de novo* inositol synthesis has on viability and lipoglycan production in *M. tuberculosis* with regard to virulence and viability in macrophages and mice is not yet understood and further work in this area is needed.

Mycothiol levels were clearly affected by inositol deprivation in the *Mtino1*Δ mutant (Movahedzadeh *et al.*, 2004). This mutant exhibited a decrease in the level of mycothiol in inositol-free medium (Fig. 1b), and it was proposed that a drop in mycothiol might make *Mtino1*Δ mutants more susceptible to the oxidative damage that occurs after the cells are phagocytosed by macrophages (Movahedzadeh *et al.*, 2004). However, a role for mycothiol in virulence is not clear. Some viable mycothiol biosynthetic mutants (*mshB* and *mshD*) are more sensitive than wild-type *M. tuberculosis* strains to oxidative damage, and do not grow well in macrophages, presumably because of a lowered resistance to oxidative damage (Newton *et al.*, 2008; Rengarajan *et al.*, 2005). In contrast to these results, another mycothiol mutant called *mshA*, which lacks detectable mycothiol, is almost fully virulent in SCID and C57BI/6 mice, suggesting that mycothiol is not required for virulence (Vilchèze *et al.*, 2008).

The actual molecular basis for the *Mtino1*Δ mutant's decrease in virulence is still unclear and much work remains to be done in order to fully understand it.

Trypanosoma brucei

Trypanosoma brucei is another microbe that must be able to generate inositol *de novo* in order to survive. Paradoxically, *T. brucei* is able to efficiently import inositol from the surrounding environment, but if it cannot synthesize inositol *de novo* it loses viability (Martin & Smith, 2006b).

The inability to obtain a homozygous *TbINO1* disruption in *T. brucei* suggested that this gene is essential. This was shown to be the case using a strain carrying a conditionally expressed allele of *TbINO1*. The two copies of *TbINO1* were disrupted only after the cell was transformed with a plasmid carrying *TbINO1* on a tetracycline-inducible promoter. The resulting strain (*INO1-myc*^{Ti}) grew only when tetracycline was present in the medium (and *TbINO1* was transcribed). When tetracycline was absent the *INO1-myc*^{Ti} strain failed to grow, indicating that *TbINO1* is essential (Martin & Smith, 2006b).

Imported inositol is used in synthesis of GPIs at only about 5 % the rate of *de novo*-generated inositol (Martin & Smith, 2006b). Metabolic labelling experiments revealed that exogenous [^{3}H]inositol was only poorly incorporated into glycolipid precursors (A and C) that are used for synthesizing the GPI anchors (Nagamune *et al.*, 2000) used to attach essential proteins like variable surface glycoprotein (VSG) to the plasma membrane (Sheader *et al.*, 2005). Since [^{3}H]inositol is very efficiently incorporated into PI in the membrane, the failure to incorporate label into VSG is not simply a matter of inadequate inositol import. In contrast, when [^{3}H]glucose is used in labelling experiments, the GPI anchor precursors (glycolipids A and C) are very efficiently labelled and incorporated into mature GPI-anchored VSGs. Thus, [^{3}H]glucose is first converted to glucose 6-phosphate, which TbIno1p then converts to inositol 3-phosphate, which is dephosphorylated to inositol that is incorporated efficiently into GPI anchors.

How can such a contradiction be explained? A model put forth by the authors suggests that there are two pools of phosphatidylinositol synthase (PIS) that tend to use inositol derived from the two different main sources: import and *de novo* synthesis. One pool of PIS is localized to the Golgi (Martin & Smith, 2006a) and uses imported inositol to generate the majority of bulk PI in the cell. The other PIS is localized to the endoplasmic reticulum (ER) (Martin & Smith, 2006a), the site where an inositol monophosphatase (IMPase) is localized (reported as unpublished results by Martin & Smith, 2006a). The authors propose that the inositol 3-phosphate synthesized

by TbIno1p is dephosphorylated at the ER by IMPase, and as a result the majority of inositol fed into the ER-localized PIS comes from inositol 3-phosphate. This ER-localized PIS is proposed to be the source of PI used for GPI anchor assembly, which also occurs in the ER. If this model is correct, then it would explain why *T. brucei* requires *TbINO1* despite its ability to import inositol. It was suggested by the authors that this mechanism may ensure that *T. brucei* always has a ready supply of inositol for the generation of VSGs. This model is summarized in Fig. 2.

The inositol transporter for *T. brucei* has not been investigated. However, work has been done on the inositol transport characteristics of the closely related parasite *T. cruzi*. *T. cruzi* possesses two inositol transport activities. One is Na^+-dependent and affects transport at high concentrations of inositol (5–10 μM), and the other is Na^+-independent and affects transport at low concentrations (4 μM and below). This suggests that *T. cruzi* has a minimum of two types of inositol transporters, at least one of which is Na^+-dependent and relies on a Na^+ gradient established across the plasma membrane. Addition of the Na^+ ATPase inhibitor furosemide partially inhibits both of these activities. Furosemide does inhibit the Na^+-dependent activity more than the 'Na^+-independent activity' (Einicker-Lamas *et al.*, 2000, 2007), but this result calls the Na^+-independence of the second activity into question. Inositol transport is energy dependent, as the addition of energy poisons such as dinitrophenol, azide and KCN also inhibit it. High concentrations of hexoses, glucose and mannose do not diminish inositol import, indicating some specificity for inositol, although this specificity has not been exhaustively examined.

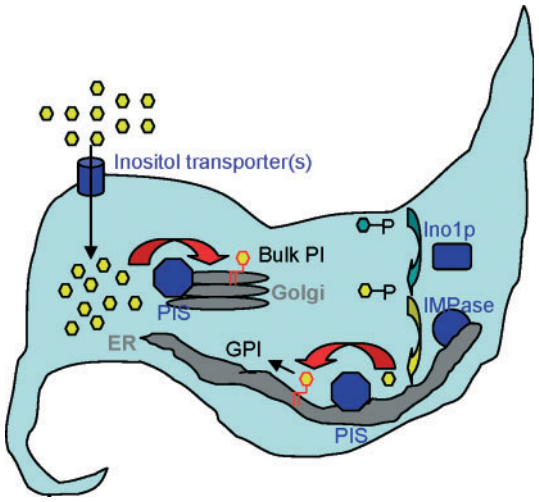

Fig. 2. The eukaryotic parasite *Trypanosoma brucei* is able to import inositol from the environment or synthesize it *de novo*. However, according to the current model, inositol imported from the environment is utilized primarily in bulk phosphatidylinositol (red and yellow phospholipid) production via a phosphatidylinositol synthase (PIS) localized to the Golgi complex. Inositol synthesized *de novo* is primarily used to generate phosphatidylinositol that is used for production of glycosylphosphatidylinositols (GPIs). The *de novo*-synthesized inositol is believed to be utilized mostly for GPI production because the IMPase that dephosphorylates inositol 3-phosphate to inositol is localized to the ER, where GPI synthesis occurs. Mutants lacking *TbINO1* are inviable because of diminished GPI production.

A number of intriguing questions remain for understanding inositol acquisition in *Trypososma* species. It remains to be seen if *T. cruzi*, like *T. brucei*, requires its *INO1* homologue for viability. Furthermore, it is not known for either of these parasites if inositol transport is required for viability or virulence. If most *de novo*-synthesized inositol is used for GPI biosynthesis, will a block in inositol transport compromise bulk PI biosynthesis and affect virulence or viability? Answering this question will require identification of the inositol transporter genes in these parasites and analysis of their function using gene deletion approaches.

Leishmania

A related kinetoplastid parasite, *Leishmania mexicana*, may share with *T. brucei* a requirement for *de novo* inositol biosynthesis for full wild-type growth and virulence. The *Lmino1*Δ mutant, which is an inositol auxotroph, exhibited poor growth even in the presence of exogenous inositol up to 2 mM (Ilg, 2002). In liquid cultures, growing as promastigotes for over 120 h, the *Lmino1*Δ mutant reached a density of only 25 % that of wild-type. The *Lmino1*Δ mutant was also avirulent in a mouse infection model. Genetic reconstitution experiments revealed that the *Lmino1*Δ::*LmINO1* reintegrate strain remained avirulent and grew to no greater density than the *Lmino1*Δ mutant in the presence of inositol, even though inositol prototrophy was restored. The reason why virulence and optimal growth were not reconstituted is not clear. It is possible that the reconstituted *LmINO1* gene is poorly expressed compared to the wild-type gene. It is also possible that the avirulence and poor growth phenotypes are genetically unlinked to the inositol auxotrophy observed in the *Lmino1*Δ mutant. While these results are not conclusive, they suggest the possibility that *L. mexicana*, like *T. brucei*, may be unable to utilize exogenous inositol efficiently for GPI synthesis. If this is the case, then utilization of exogenous inositol appears to be more efficient in *L. mexicana* than *T. brucei*, since *L. mexicana* exhibits some limited growth. This is an area for further investigation.

Despite potential functional similarities in the *INO1* requirement of *Leishmania* and *Trypanosoma* species, the inositol transporters appear to be quite different. In *Leishmania donovani*, inositol is transported by a single H^+-dependent inositol symporter that relies on the H^+ gradient across the plasma membrane to supply its energetics. The *L. donovani myo*-inositol transporter (MIT) has been cloned, and its predicted structure is very

similar to that of the sugar transporter family (Drew *et al.*, 1995; Jin & Seyfang, 2003; Seyfang & Landfear, 2000). MIT may be necessary for full virulence, as a disruption of the *L. dononvani MIT* gene resulted in a mutant that grew at a reduced rate. However, its virulence has not been analysed in an animal model (Mongan *et al.*, 2004).

Despite its sequence similarity to sugar transporters, MIT is very specific for inositol and does not appear to recognize sugar molecules of similar structure such as glucose, galactose, mannose, fucose, xylose and others (Mongan *et al.*, 2004). It has been suggested that it may be possible to exploit this specificity to design toxic analogues of inositol as antibiotics, since the specificities of the *Leishmania* MIT and the human Na^+/inositol transporters SMIT1 and SMIT2 are different. For example, SMIT1 recognizes fucose (Hager *et al.*, 1995), and SMIT2 recognizes glucose and xylose (Coady *et al.*, 2002). However, a complete one-to-one comparison of substrate specificities for the human H^+/inositol transporter (HMIT; Uldry *et al.*, 2001) and *L. donovani* MIT has not been performed. Nonetheless, an exciting possibility is that if there is a difference between the specificities of MIT and HMIT, then it may be possible to create toxic analogues of inositol that will specifically be taken up by *Leishmania* MIT and not by human inositol transporters.

Other parasites

A potential contrast to the above-mentioned parasites may exist among the apicomplexan parasites *Toxoplasma gondii* and *Cryptosporidium parvum*. BLAST searches against the whole-genome sequences of these parasites at EUPath (http://eupathdb.org/eupathdb/) failed to reveal a homologue for Ino1p. It is possible that *T. gondii* and *Cryptosporidium* do have Ino1p homologues that were missed in the BLAST search algorithm, or they may have functional homologues that differ in primary sequence. However, if indeed these pathogens cannot make inositol *de novo*, then they must have mechanisms for acquiring inositol from the host. This would make inositol transporters essential for these pathogens, which would be an interesting contrast to the case of *Trypanosoma brucei*, where *de novo* synthesis is essential.

Candida albicans

Candida albicans appears to be much more versatile than the above pathogens. During a bloodstream infection it is able to cause infection with equal efficiency whether it imports inositol or makes it *de novo* (Chen *et al.*, 2008). A *C. albicans Caino1*Δ/*Caino1*Δ mutant is an inositol auxotroph. However, unlike *T. brucei*, *L. mexicana* or *M. tuberculosis*, *C. albicans Caino1*Δ/*Caino1*Δ mutants are as virulent as wild-type in a mouse model of systemic infection. These data initially suggested two possibilities: (1) *C. albicans* needs to transport inositol rather than make it *de novo* to support an infection; (2) *C. albicans* can synthesize or transport inositol with equal efficiency to support an infection.

C. albicans possesses a high-affinity inositol transporter that, like the *L. donovani* transporter, is dependent on a proton gradient across the membrane (Jin & Seyfang, 2003). Biochemical analysis of the transport kinetics suggests that there is only one transporter that is specific for *myo*-inositol, and it will not recognize glucose, galactose, mannose, fructose, fucose, arabinose and xylose. Based on homology to the *S. cerevisiae* inositol transporters ScItr1p and ScItr2p (Nikawa *et al.*, 1991), the *C. albicans* transporter was identified and cloned, and the gene was disrupted (Chen *et al.*, 2008). The *Caitr1*Δ/*Caitr1*Δ mutant lacked inositol transport activity compared to the wild-type strain, indicating that there is only one transporter, as the previous kinetic studies had suggested (Jin & Seyfang, 2003). Despite the lack of inositol transport, the *Caitr1*Δ/*Caitr1*Δ mutant is fully virulent in a mouse model of systemic infection.

These results indicate that *C. albicans* is able to acquire sufficient inositol to support an infection whether it synthesizes inositol *de novo* or imports it from the host. The serum of rats and humans contains 20–100 μM and 61 ± 12.4 μM inositol, respectively (Isaacks *et al.*, 1997; Kouzuma *et al.*, 2001; Palmano *et al.*, 1977). Mouse serum is most likely similar, as the *Caino1*Δ/*Caino1*Δ mutant can clearly proliferate in the mouse (Chen *et al.*, 2008).

In order to determine if CaIno1p and CaItr1p provide the only two routes for acquiring inositol from the host, a conditional double mutant between *Caino1*Δ and *Caitr1*Δ was constructed. In *C. albicans* the *MET3* promoter (P_{MET3}) can be used as a conditional promoter because P_{MET3} is strongly activated in the absence of sulfur-containing amino acids such as cysteine and methionine (Cys/Met), but in the presence of Cys/Met in the medium P_{MET3} is transcriptionally repressed (Care *et al.*, 1999). A conditional double mutant was constructed by disrupting both copies of *CaINO1* (*Caino1*Δ/*Caino1*Δ) and one copy of *CaITR1* (*Caitr1*Δ). The promoter of the remaining wild-type copy of *CaITR1* was replaced on the chromosome with the P_{MET3} promoter. The resulting strain grew at a wild-type rate in the absence of Cys/Met in the medium, but did not grow at all in the presence of Cys/Met. These data indicated that *C. albicans* has no way to obtain inositol *in vitro* aside from *de novo* synthesis through CaIno1p or import via CaItr1p. This appears to be true for *C. albicans* in the host as well, since in a mouse model for systemic infection, the conditional double mutant was avirulent and the mice showed no symptoms of infection, which is consistent with the hypothesis that the strains could not survive due to a lack of inositol. The mouse bloodstream contains sufficient Cys/Met to shut off the P_{MET3} promoter (Chen *et al.*, 2008; Rodaki *et al.*, 2006). Even a strain that contained homozygous mutations for *CaINO1* and was heterozygous for *CaITR1* was attenuated for virulence compared to the wild-type, although it was more virulent than the conditional double mutant.

These data indicate that *C. albicans*, unlike *M. tuberculosis* and *T. brucei*, is able to acquire inositol by importing it from the host or by synthesizing it *de novo* (Fig. 3), and either mechanism is sufficient to cause a wild-type infection. The reasons for these differences in strategies are unclear, but one large difference between *M. tuberculosis* and *T. brucei* and *C. albicans* is that *C. albicans* is a commensal organism. It is normally found in the gut, oral and/or vaginal tracts of humans (Calderone, 2002). Its versatility may help it grow in many different host environments. However, this hypothesis has yet to be tested, as these mutants have not been examined in the context of vaginal, oral or gut infection models. In addition, even in the mouse bloodstream infection model it is not clear which mechanism wild-type *C. albicans* prefers to use. For example, does *C. albicans* normally use both mechanisms during an infection or does it favour one method when growing as a commensal and the other during invasive disease? Is there tissue-specific expression of these genes so that the method of inositol acquisition depends on whether the organism grows in the mucosa, the bloodstream or deep organs? All of these questions remain to be answered.

How and when CaItr1p and CaIno1p are expressed in the host will be best understood when regulators of these genes are identified. Although *S. cerevisiae* is often a useful guide for exploring such questions in *C. albicans*, in this case there appears to be transcriptional rewiring between these two yeasts regarding the regulation of their respective Ino1p homologues. Thus, CaIno1p regulation is an area of research where much remains to be learned.

The *S. cerevisiae* transcriptional regulators of *ScITR1* and *ScINO1* are well known. Two proteins, ScIno2p and ScIno4p, form a heterodimeric transcriptional activator of *ScITR1* and *ScINO1* as well as other phospholipid biosynthetic genes. The protein ScOpi1p acts as a repressor of these same targets by binding to ScIno2p (reviewed by Chen *et al.*, 2007; Greenberg & Lopes, 1996). Recent studies have revealed that the *C. albicans* sequence homologues of ScIno2p and ScIno4p (CaIno2p and CaIno4p) do not appear to regulate *CaINO1*, and may regulate ribosomal genes (Hoppen *et al.*, 2007). It was even suggested that *CaINO2* and *CaINO4* are essential, although these studies did not conclusively demonstrate this. In addition, CaOpi1p (a sequence homologue of ScOpi1p) does not repress transcription of *CaINO1*. A *Caopi1*Δ*/Caopi1*Δ mutant represses *CaINO1* in response to exogenous inositol levels in the same manner as a wild-type strain (Y. L. Chen & T. B. Reynolds, unpublished data). This is despite the fact that CaOpi1p can complement a *Scopi1*Δ mutant for regulation of *ScINO1* when expressed heterologously in *S. cerevisiae* (Heyken *et al.*, 2003). Thus, the actual regulators of *CaINO1*, *CaITR1* and other *C. albicans* phospholipid biosynthetic genes remain to be identified. Identification of basic regulatory components for phospholipid biosynthesis in *C. albicans* will aid in understanding the regulation of CaIno1p and CaItr1p during an infection.

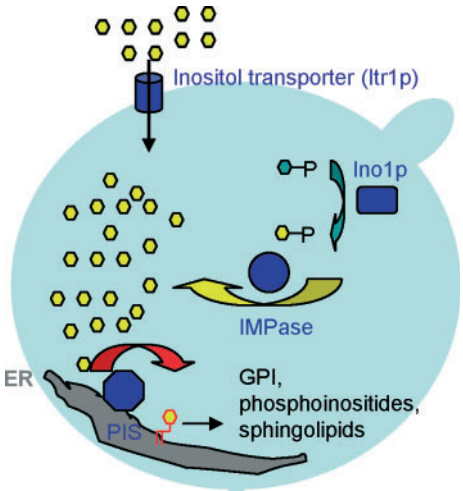

Fig. 3. The fungal pathogen *Candida albicans* can acquire inositol by *de novo* synthesis or by importing it, and either mechanism is sufficient to support wild-type growth *in vitro* and full virulence.

It is not known why *C. albicans* mutants lose viability when deprived of inositol, but it is very likely due to the pleiotropic loss of PI and its downstream products, including GPI anchors (Doering & Schekman, 1996), membrane phosphoinositides (Strahl & Thorner, 2007) and sphingolipids (Dickson & Lester, 1999). However, it remains to be determined precisely what defects cause the loss of viability during infection.

Cryptococcus neoformans

Cryptococcus neoformans is a fungal pathogen that causes a fatal meningoencephalitis especially in immunocompromised patients such as those with acquired immune deficiency syndrome (AIDS) (Casadevall & Perfect, 1998). *Cryptococcus* species such as *C. neoformans* and *C. gattii* are believed to enter the host through the inhalation of spores or desiccated yeast cells that are produced in the environment. The known environmental niches of *C. neoformans* and *C. gattii* are pigeon guano and eucalyptus trees, respectively (Casadevall & Perfect, 1998; Idnurm *et al.*, 2005). This led to the hypothesis that *Cryptococcus* species might sporulate in association with plants or trees, and it was recently demonstrated that substances extracted from plants can stimulate mating and sporulation in both *C. neoformans* and *C. gattii* (Xue *et al.*, 2007). Interestingly, the substance with the strongest effect on mating and sporulation was inositol, which is abundant in plants. If spore formation by *Cryptococcus* species is a major source of infectious propagules for humans, then inositol may be an environmental cue that promotes human infections by inducing mating and sporulation.

The mechanism by which inositol promotes mating and sporulation in *Cryptococcus* species is not known, but other

observations suggest that *Cryptococcus* species need to acquire a relatively large amount of inositol. *C. neoformans* not only generates inositol through a functional inositol-3-phosphate synthase (CnIno1p), it also catabolizes inositol as a sole carbon source via an inositol oxygenase (IOase) activity (Kanter *et al.*, 2003; Molina *et al.*, 1999). Although some *Cryptococcus* species repress their IOase activity in the presence of glucose as a carbon source (Kanter *et al.*, 2003), *C. neoformans* expresses IOase activity even when glucose is present in the medium, and the IOase activity increases when inositol is the sole carbon source (Molina *et al.*, 1999). The CnIno1p activity of *C. neoformans* is much higher than that of *S. cerevisiae*. For example, in *C. neoformans* the CnIno1p activity is about sevenfold greater than that of *S. cerevisiae* when each strain is grown in medium containing glucose, and lacking inositol. When excess inositol is added to the medium (75 μM), the ScIno1p activity is reduced to undetectable levels in *S. cerevisiae*, but in *C. neoformans* the CnIno1p activity level is similar to that of *S. cerevisiae* in the absence of inositol. Only when inositol is used as the sole carbon source is CnIno1p activity reduced to undetectable levels (Molina *et al.*, 1999). Bioinformatic analysis predicts that *C. neoformans* has approximately seven inositol transporters (Xue *et al.*, 2007). Altogether these data suggest that *C. neoformans* has a strong requirement for inositol.

The reasons why *C. neoformans* has such a strong requirement for inositol are unclear. In plants inositol is converted by an IOase activity to glucuronic acid, which feeds into both the pentose phosphate pathway and the UDP-glucuronic acid pathway for cell wall biosynthesis (Loewus *et al.*, 1962; Seitz *et al.*, 2000; Tenhaken & Thulke, 1996). In *C. neoformans* UDP-glucuronic acid is important for manufacturing an important virulence factor, the capsule (Bar-Peled *et al.*, 2004; Griffith *et al.*, 2004; Moyrand & Janbon, 2004). It has been postulated that the IOase activity in *C. neoformans* is supplying glucuronic acid used to make UDP-glucuronic acid for capsule biosynthesis (Kanter *et al.*, 2003). However, this does not appear to be the case, as two different studies have shown that mutants carrying disruptions of the *UGD1* gene, which encodes the UDP-glucose dehydrogenase that converts UDP-glucose to UDP-glucuronic acid, completely lack UDP-glucuronic acid and capsule synthesis (Griffith *et al.*, 2004; Moyrand & Janbon, 2004). This indicates that in *C. neoformans* the IOase does not supply UDP-glucuronic acid for capsule biosynthesis. It is possible that IOase does generate glucuronic acid for the pentose phosphate pathway (Hankes *et al.*, 1969; Kanter *et al.*, 2003; Prabhu *et al.*, 2005). Whether CnIno1p, IOase or the many inositol transporter homologues in *C. neoformans* play a role in virulence in humans is unclear. The role that inositol plays in stimulating mating in *C. neoformans* and its virulence as a dikaryon in plants (Xue *et al.*, 2007) is also not understood.

C. neoformans has a tropism for the central nervous system, which is rich in inositol (Fisher *et al.*, 2002; Heitman *et al.*, 2006). Tropism for the brain could be driven by the strong requirement for inositol (Molina *et al.*, 1999). It has also been suggested that brain tropism is driven by the abundance of catecholamines in the brain, which can serve as precursors for biosynthesis of melanin, another virulence factor (Eisenman *et al.*, 2007; Polacheck *et al.*, 1990). These factors are not necessarily mutually exclusive. Much work remains to be done to understand if there is a connection between *Cryptococcus* inositol metabolism and brain tropism.

Prospectus

The broad range of mechanisms by which pathogens synthesize phospholipids during an infection, and the surprising strategies that pathogens use to acquire inositol for PI synthesis while in the host, demonstrate that this area of research will reveal many fascinating aspects of host–pathogen biology. It is interesting that two very different pathogens, *Trypanosoma brucei* and *Mycobacterium tuberculosis*, one an extracellular eukaryotic parasite and the other an intracellular prokaryotic parasite, both require inositol biosynthesis for survival and pathogenesis, respectively. The reasons for this requirement, however, are quite different. In contrast, the fungal pathogen *Candida albicans* requires neither synthesis nor import exclusively, but can survive using either. These studies raise many questions about inositol acquisition in the host. Is the requirement for *de novo* inositol biosynthesis found in other mycobacterial pathogens such as *M. leprae* and *M. ulcerans?* Do other parasites besides *T. brucei* require *de novo* inositol biosynthesis for viability or virulence? Do the apicomplexan parasites *Toxoplasma gondii* and *Cryptospordium parvum* carry functional *INO1* homologues? Do other fungal pathogens besides *C. albicans* exhibit versatility in their acquisition of inositol?

Another area for future inquiry is whether any of the enzymes involved in inositol biosynthesis or import may be useful drug targets. For example, could the requirement for the Ino1p enzyme be exploited to generate novel antimicrobials for *T. brucei* or *M. tuberculosis*? This enzyme is admittedly not ideal because a homologue exists in the host. However, it may be possible to identify a drug that exhibits specificity for the parasite or bacterial enzyme.

CaIno1p clearly cannot serve as a drug target in *C. albicans*. However, there are other enzymes in this fungal pathogen involved in phospholipid biosynthesis that may be more promising. For example, the phosphatidylserine synthase (*CaCHO1*) of *C. albicans* is unique to fungi and not found in humans (Braun *et al.*, 2005). This has been suggested as a useful drug target. Similarly, enzymes involved in phosphatidylcholine biosynthesis have been suggested as potential antimicrobial drug targets in *Plasmodium falciparum* (Witola *et al.*, 2008). Phospholipid biosynthesis in these pathogens appears to be a promising area for exploration.

Acknowledgements

I would like to thank Dr Neil Stoker, Dr Terry Smith, Dr Joseph Heitman, Dr Chaoyang Xue and especially Dr Pamela Small for their insightful comments and helpful discussions regarding this manuscript. This work was supported by grants AHA 0765366B and NIH-1R03AI071863-01A1.

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