



Role of phospholipases in fungal fitness, pathogenicity, and drug development – lessons from *Cryptococcus neoformans*

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Many pathogenic microbes, including many fungi, produce phospholipases which facilitate survival of the pathogen *in vivo*, invasion and dissemination throughout the host, expression of virulence traits and evasion of host immune defense mechanisms. These phospholipases are either secreted or produced intracellularly and act by physically disrupting host membranes, and/or by affecting fungal cell signaling and production of immunomodulatory effectors. Many of the secreted phospholipases acquire a glycosylphosphatidylinositol sorting motif to facilitate membrane and/or cell wall association and secretion. This review focuses primarily on the role of two members of the phospholipase enzyme family, phospholipase B (Plb) and phosphatidylinositol (PI)-specific phospholipase C (PI-C/Plc), in fungal pathogenesis and in particular, what has been learnt about their function from studies performed in the model pathogenic yeast, *Cryptococcus neoformans*. These studies have revealed how Plb has adapted to become an important part of the virulence repertoire of pathogenic fungi and how its secretion is regulated. They have also provided valuable insight into how the intracellular enzyme, Plc1, contributes to fungal fitness and pathogenicity – via a putative role in signal transduction pathways that regulate the production of stress-protecting pigments, polysaccharide capsule, cell wall integrity, and adaptation to growth at host temperature. Finally, this review will address the role fungal phospholipases have played in the development of a new class of antifungal drugs, which mimic their phospholipid substrates.

Keywords: *Cryptococcus neoformans*, phospholipase B, phosphatidylinositol-specific phospholipase C, PI-PLC/Plc, pathogenicity, drug development, secretion, signaling

THE PHOSPHOLIPASE ENZYME FAMILY AND THE ACTIVITIES OF FUNGAL PLB AND PLC

Phospholipases are a heterogeneous group of enzymes that hydrolyse one or more ester linkages in glycerophospholipids (Figure 1), which are the major components of cell membranes and lung surfactant. Each glycerophospholipid consists of a glycerol backbone containing one (lysophospholipid) or two (phospholipid) ester linkages to hydrophobic fatty acid chains, and a third ester linkage to a phosphate head group. The phosphate head group is also linked by an ester bond to a variety of polar moieties, such as choline, ethanolamine, serine, and inositol. Phospholipases are categorized into five groups depending on which ester bond is hydrolysed: A₁, A₂, B, C, and D (Figure 1). Two major phospholipases associated with pathogenicity in fungi are phospholipase B (Plb) and phosphatidylinositol (PI)-preferring phospholipase C (PI-PLC/Plc). Plb enzymes remove both fatty acyl chains from glycerophospholipids. They may also have lysophospholipase (LPL) and lysophospholipase-transacylase (LPTA) activities, both of which can contribute to the detoxification of potentially cellulolytic lysophospholipids. Specifically, the LPL activity removes the single fatty acyl chain on a lysophospholipid to produce glycerophosphocholine (GPC) and the LPTA activity re-esterifies an acyl chain onto a lysophospholipid, thus regenerating a glycerophospholipid (Figure 2). It has also been proposed that PLB activity is, in fact, a combination of phospholipase A₂ (PLA₂) and LPL activities. PI-PLCs (or Plcs)

remove the polar head group from PI, which can be present as either mono- or diphosphorylated inositol or as the glycosylphosphatidylinositol (GPI) anchor on some membrane and cell wall proteins, including fungal Plbs (Figure 1). Both Plb and PI-PLC can be secreted or remain intracellular. Many Plb enzymes have a GPI anchor which provides an additional level of secretory regulation and appears to serve as a sorting signal for efficient cellular export of fungal Plbs. The lipid derived products of Plb and PI-PLC may act as secondary messengers in signal transduction, or exert immunomodulatory effects.

CRYPTOCOCCUS NEOFORMANS – AN EFFECTIVE MODEL FUNGAL PATHOGEN FOR CHARACTERIZING PHOSPHOLIPASE VIRULENCE DETERMINANTS

The basidiomycetous fungal pathogen, *Cryptococcus neoformans*, has a predilection to cause infection in patients with AIDS and is responsible for an estimated one million cases of meningitis and 675,000 deaths per year in HIV-infected patients alone (Park et al., 2009). Infections also occur in immunocompetent individuals (Chau et al., 2010). The other pathogenic *Cryptococcus* species, *C. gattii*, mainly infects immunocompetent individuals and was responsible for an outbreak of cryptococcosis that was first noted in 1999 on Vancouver Island, British Columbia (BC), Canada (Ma et al., 2007). There are two varieties of *C. neoformans*, *C. neoformans var. grubii* (Serotype A) and *C. neoformans var.*

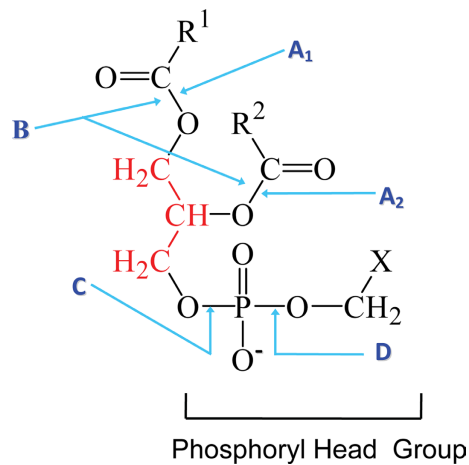


FIGURE 1 | The phospholipid cleavage sites of phospholipase enzymes.

The phospholipid glycerol backbone is shown in red. Phospholipase A₁ (A₁) cleaves a fatty acyl ester at the *sn1* position while phospholipase A₂ (A₂) removes the fatty acid at the *sn2* position to yield a lysophospholipid and a fatty acid. Phospholipase B (B) removes both acyl chains from the *sn1* and *sn2* positions on the glycerol backbone. The phosphodiester bond in the head group proximal to the glycerol backbone is cleaved by phospholipase C (C) to yield diacylglycerol and the phosphoryl head group. PI-PLC enzymes perform this cleavage when X is inositol, which may, or may not, be linked to phosphates or a sugar chain composed predominantly of mannosyl sugars in the case of a GPI anchor. Cleavage by phospholipase D (D) at the phosphodiester bond proximal to X, yields phosphatidic acid and X, which can be either ethanolamine, serine, inositol, or choline. Adapted from the PhD thesis of Dr Rosemary A. Sifakas.

neoformans (Serotype D), with serotype A being the more clinically prevalent and virulent serotype (Martinez et al., 2001). The predominant natural habitat of *C. neoformans* is nitrogen-rich avian guano, soil and possibly decaying material from trees. It has been suggested that its pathogenicity for mammals evolved as a consequence of phagocytic interactions with soil amoeba (Alvarez et al., 2009). In immunocompetent individuals, phagocytosis of inhaled spores or small desiccated yeast cells by macrophages may result either in clearing of the infection or establishment of a latent infection in the lung. However, in the immunocompromised individual there is evidence that macrophages may also transport *C. neoformans* to the central nervous system where it establishes severe life-threatening meningoencephalitis (Charlier et al., 2009). Not only is *C. neoformans* an important pathogen in its own right, it is also an excellent model for studying fungal pathogenesis, as the recently sequenced haploid genomes of both serotypes A and D are amenable to a range of genetic manipulation techniques, including targeted gene disruption and RNAi and robust animal models of infection are available to assess the role of genes in virulence. Much of our knowledge about fungal phospholipase enzymatic activities and their contribution to pathogenesis has been derived from studies performed on cryptococcal enzymes. In fact, *C. neoformans* is the only fungal pathogen in which the effect of absence of the Plc1-encoding gene on fungal fitness and pathogenicity has been determined (Chayakulkeeree et al., 2008).

PHOSPHOLIPASE B ENZYMES IMPLICATED IN FUNGAL VIRULENCE

It is well-established through gene deletion and reconstitution experiments that phospholipases contribute to the virulence repertoire of pathogenic fungi, despite their presence in the mainly non-pathogenic and industrial yeasts. Pathogenic fungi that secrete phospholipase B include *C. neoformans* (Chen et al., 1997b; Santangelo et al., 1999), *Candida albicans* (Ibrahim et al., 1995; Mirbod et al., 1995; Theiss et al., 2006), and *Aspergillus fumigatus* (Shen et al., 2004). The most well characterized phospholipase B enzymes produced by fungal pathogens are phospholipase B 1, 2, and 5 from *C. albicans* (Sugiyama et al., 1999; Mukherjee et al., 2001; Theiss et al., 2006) and phospholipase B1 from *C. neoformans* (Chen et al., 2000) and *Cryptococcus gattii* (Wright et al., 2004) (CaPlb1/2/5, CnPlb1, CgPlb1, respectively). Only CaPlb2 does not play a significant role in virulence while the role of CgPlb1 in virulence has not been assessed. CnPlb1, CaPlb1, and CgPlb1 all possess PLB, LPL and LPTA enzymatic activities, with properties that differ to each other and to Plb1 homologs produced by industrial yeasts (Takahashi et al., 1991; Mirbod et al., 1995; Chen et al., 2000).

The *C. neoformans* genome contains a single *PLB* gene, *PLB1*, with an established role in virulence (Cox et al., 2001). In addition to CnPlb1, *C. neoformans* produces a Plb1-independent phospholipase A1, which degrades 1-palmitoyl-2-arachidonoyl phosphatidylcholine. Neither the gene encoding this enzyme, nor the protein it specifies, have been isolated (Wright et al., 2007) and its role in virulence has not been determined. *C. neoformans* also produces two additional LPL/LPTA enzymes, *CnLyso1* and *CnLpl1*, which were characterized in serotype D (Coe et al., 2003) and *C. gattii* (Wright et al., 2004), respectively. The *CnLYSO1* found in serotype D itself is not predicted to be secreted as it has no secretory leader peptide, but appears to affect the secretion of CnPlb1 (Coe et al., 2003). Its role in virulence is unknown. Interestingly, although CnLpl1 found in serotype A does contain a putative secretory leader peptide, it does not contribute to the pool of secreted PLB activity, at least in cultures maintained on agar; this pool was attributed solely to CnPlb1 activity (Cox et al., 2001). Disruption of the *CnLPL1* homolog in serotype A altered neither growth nor virulence of *C. neoformans* in a mouse model (M. Chayakulkeeree, and J. T. Djordjevic, unpublished observation). It may function in association with CnLyso1 to remove toxic lysophospholipids (Wright et al., 2007). These three enzymes may provide functional redundancy by maintaining cryptococcal growth in the absence of CnPlb1.

The genome of *C. albicans* encodes a Plb multigene family with five putative members. The best studied genes in this family are *CaPLB1*, *CaPLB2*, and *CaPLB5* which encode putative secreted proteins (Leidich et al., 1998; Sugiyama et al., 1999; Mukherjee et al., 2001; Theiss et al., 2006). Only *CaPLB1* and *CaPLB5* have an established role in virulence (Leidich et al., 1998; Hoover et al., 1998; Mukherjee et al., 2001; Theiss et al., 2006). Abrogation of CaPlb1 and CaPlb5 enzymatic activity by inactivation of *CaPLB1* and *CaPLB5*, respectively, rendered the mutant strains less virulent in systemic mouse models of infection as measured by host tissue colonization (Leidich et al., 1998; Mukherjee et al., 2001; Theiss et al., 2006). Loss of these genes coincided with reduced LPTA/

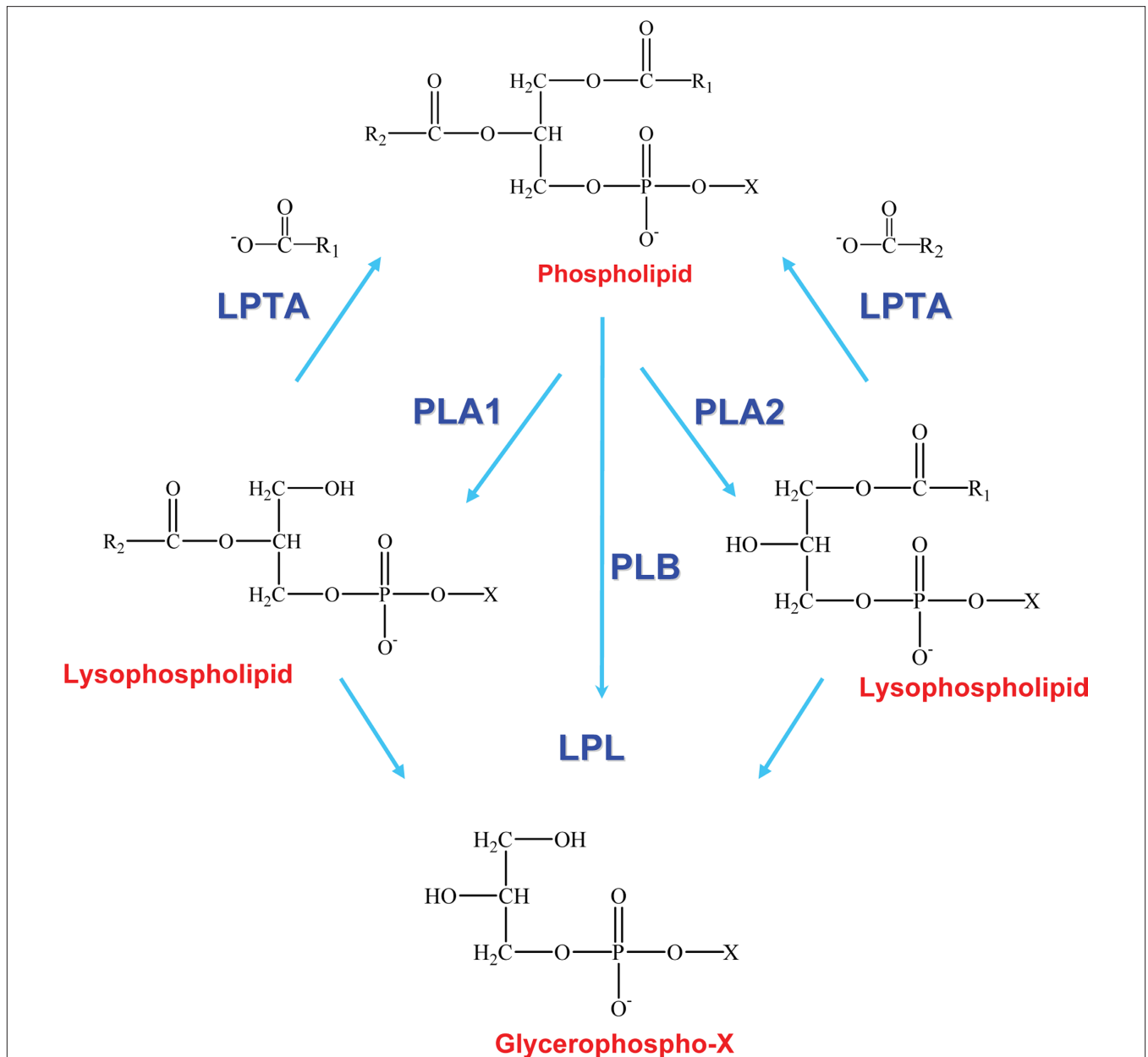


FIGURE 2 | Enzymatic activities of phospholipase B enzymes.

Phospholipase B (PLB) activity is a combination of PLA₁ and PLA₂ activities (or PLA and LPL activity) and generates glycerophospho compounds. Lysophospholipid, a short-lived intermediate, is also a substrate for lysophospholipase (LPL) and lysophospholipase-transacylase (LPTA)

activities, which rapidly hydrolyse or re-esterify lysophospholipid, respectively. R1 and R2 represent fatty acid chains in the *sn1* and *sn2* position of the phospholipid, respectively. X refers to the head group (e.g., serine, ethanolamine, choline, or inositol). Adapted from the PhD thesis of Dr Rosemary A. Siafakas.

PLB and PLA₂ activity, respectively. Neither the role of CnPlb1 and CaPlb5 in virulence nor their enzymatic properties have been determined. The genome of *A. fumigatus* encodes three *PLB* genes (Shen et al., 2004). However, their role in virulence has not yet been established. It is important to note that not all pathogenic fungi secrete significant amounts of Plb. One such example is *Candida parapsilosis* where, due to contradictory findings among various investigators, the role of secreted Plb in pathogenesis is unclear (Trofa et al., 2008).

PLB HOMOLOGS IN PATHOGENIC FUNGI DIFFER FROM THOSE IN NON-PATHOGENIC FUNGI

PLB genes are present in non-pathogenic yeasts including *Saccharomyces cerevisiae*, *Debaryomyces (Torulaspora) hansenii*, *Penicillium notatum*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Neurospora crassa*, and *Kluyveromyces lactis* (reviewed in Ghannoum, 2000). Extensive enzymatic characterization of the Plb enzymes secreted by the pathogenic yeasts, *C. neoformans* and *C. albicans*, and a comparison of their properties to the Plb

homologs produced by non-pathogenic yeasts, has revealed why these enzymes are more adapted to a role in virulence (Chen et al., 2000). Firstly, the specific activities of purified cryptococcal and candidal Plb1 enzymes are 10- to 200-fold higher than the corresponding homologs from non-pathogenic fungi (Lee et al., 1994; Mirbod et al., 1995; Merkel et al., 1999; Chen et al., 2000). Secondly, the major membrane phospholipids, dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol, which are also the most abundant components of lung surfactant, and lysophosphatidylcholine, are preferred substrates of CnPlb1, whereas the less accessible, acidic inner-leaflet phospholipids, phosphatidylserine, phosphatidylinositol, and phosphatidic acid are preferred substrates of Plbs from non-pathogenic fungi (Witt et al., 1984; Saito et al., 1991; Oishi et al., 1996; Merkel et al., 1999). This observation suggests that Plb1 secreted by pathogenic fungi is more likely to digest host cell membranes and surfactant than Plb1 secreted by non-fungal pathogens. In support of this, the extent of cell damage caused by culture supernatants prepared from *C. albicans* (62%) was less than that caused by the *CaPLB1*-deficient strain and *S. cerevisiae* (35 and 4%, respectively) as assessed using a thiazolium blue-based assay (Ghannoum, 2000). Thirdly, a range of protein modifying agents differentially affect the activities of CnPlb1, Plb homologs from non-pathogenic fungi and mammalian secretory PLA₂ (Chen et al., 2000). The millimolar quantities of acetic acid secreted by *C. neoformans in vitro* (Bubb et al., 1999) and in experimental lung and brain cryptococcomas (Himmelreich et al., 2003) may create a microenvironment favorable for CnPlb1, which has an acidic pH optimum (Chen et al., 2000). As *C. neoformans* is well-adapted to intracellular survival in the acidified phagolysosome (pH 5.0) of human macrophages (Levitz et al., 1999), secreted CnPlb1 may be a contributing survival factor.

EVIDENCE FOR PRODUCTION OF FUNGAL PLB *IN VIVO*

There is evidence to suggest that Plb1 produced by pathogenic fungi is also produced during infection *in vivo*. CaPlb1 was identified immunologically in host tissue (Ghannoum, 2000; Mukherjee et al., 2001) and the GPC product of CnPlb1 is present in lung and brain cryptococcomas produced in a rat model (Himmelreich et al., 2003; Sorrell et al., 2006). Furthermore, antibodies to CaPlb1 and CnPlb1 have been identified in the serum of infected patients (Ghannoum, 2000; Santangelo et al., 2005). In addition, blood isolates of *C. albicans* generally secrete much higher levels of CaPlb1 than isolates from the oral cavity of healthy individuals (Ibrahim et al., 1995) or isolates from wounds or urine (Price et al., 1982).

FACTORS REGULATING CNPLB1 CELLULAR EXPORT TO THE PERIPHERY AND SECRETION

Cryptococcal and candidal Plbs with an established role in virulence (CnPlb1, CaPlb1, CaPlb2, and CaPlb5) contain a secretory leader peptide at the N-terminus and we have confirmed that deletion of this motif in CnPlb1 abolishes its secretion within a heterologous expression system (Djordjevic et al., 2005). This motif permits entry of nascent Plb into the endoplasmic reticulum (ER) where it is folded and acquires asparagines (N)-linked glycosylation which in the case of CnPlb1 is essential for activity (Chen et al., 2000) and export and secretion (Turner et al., 2006). The Plbs are then

transported to the surface and subsequently secreted. Both CnPlb1 and CaPlb1 have been detected in fungal secretions and within host tissue. In the case of CnPlb1, secretion still occurs despite the acquisition a glycosylphosphatidylinositol (GPI) anchor which permits CnPlb1 association with membranes and the cell wall, suggesting that CnPlb1 must be released from the anchor prior to secretion. In contrast to CnPlb1, CaPlb1, and CaPlb2 do not acquire a GPI anchor, while CaPlb5 (and CaPlb3, CaPlb4) are predicted to acquire one (Theiss et al., 2006). Of the Plbs produced by *A. fumigatus*, AfPlb2, is the only Plb not predicted to be secreted or GPI anchored. It is not known why some Plbs involved in virulence acquire a GPI anchor and some do not. However, in addition to permitting membrane and cell wall localization, the anchor in CnPlb1 appears to play a role in regulating CnPlb1 export and secretion.

GPI anchors and rafts. Following export through the secretory pathway to the cell periphery, some fungal GPI-anchored proteins are retained at the plasma membrane while others are released from it by an undefined cleavage mechanism within the GPI anchor (Lu et al., 1994; Kollar et al., 1997; Fujii et al., 1999; Gagnon-Arsenault et al., 2008). Cleavage allows the GPI-anchored protein to be transported to the cell wall and covalently cross-linked to β -1,6-glucans via the GPI anchor remnant (Kapteyn et al., 1994; Lu et al., 1994) or secreted. The means by which fungal GPI-anchored proteins are differentially targeted to the membrane or cell wall are poorly understood. However, the signals reside in the C-terminus (Mao et al., 2008). We have found CnPlb1 to be associated with both membranes and the cell wall fraction (Siafakas et al., 2006; Siafakas et al., 2007).

In fungi, GPI-anchored proteins cluster within sphingolipid/sterol-rich microdomains, or rafts, within the plasma membrane (Muniz and Riezman, 2000; Insenser et al., 2006). We also demonstrated that treatment of cryptococci with the raft disrupting agent, methyl- β -cyclodextrin, which binds to sterols, releases CnPlb1 protein and enzyme activity into the external milieu, consistent with CnPlb1 also localizing within plasma membrane rafts following its transport to the cell periphery (Siafakas et al., 2006). We also found that CnPlb1-containing membranes are enriched in sterols, insoluble in Triton X-100 at 4°C and of a higher buoyant density than Triton X-100-soluble membranes (Siafakas et al., 2006), consistent with the properties of rafts (Bagnat et al., 2000). We proposed that raft membranes serve to cluster CnPlb1 at the cell surface to allow efficient access to enzyme substrate and/or to provide a mechanism for rapid release to the external environment (Siafakas et al., 2006). However, since both raft assembly and the sorting of GPI anchored proteins into raft-enriched secretory vesicles occurs within the ER (Sutterlin et al., 1997; Bagnat et al., 2000; Muniz and Riezman, 2000), the association of CnPlb1 with cryptococcal rafts may also be an early secretory event.

GPI anchors are also essential for export of CnPlb1 to the periphery as CnPlb1 export is blocked when GPI anchor synthesis is blocked (Siafakas et al., 2007). In addition, deletion of the GPI anchor consensus motif, which serves as a temporary ER retention signal until fully synthesized GPI anchors are substituted for it, resulted in a higher ratio of secreted to cell-associated CnPlb1 in a heterologous *S. cerevisiae* expression system due to the lack of CnPlb1 retention in the membrane and cell wall (Djordjevic et al.,

2005). However despite this, absence of a GPI anchor resulted in a greater than 50% reduction in the rate of CnPlb1 export and secretion, consistent with export of truncated protein being less efficient than that of native CnPlb1 (Djordjevic et al., 2005). Thus, the acquisition of the GPI anchor in CnPlb1 serves to expedite CnPlb1 transport through the secretory pathway, and to localize CnPlb1 at the fungal cell periphery.

N-linked Glycosylation. Using site-directed mutagenesis, we also showed that altering just a single N-linked glycosylation site out of the potential 17 that exist in CnPlb1, caused a retardation in cellular export and secretion of CnPlb1 within the heterologous host, *S. cerevisiae*, irrespective of GPI anchor status, consistent with N-linked glycosylation being more crucial for CnPlb1 traffic than acquisition of the GPI anchor. Specifically, CnPlb1 export and secretion was compromised when three individual glycosylation sites, Asn56, Asn430, and Asn550, were changed to Ala (Turner et al., 2006).

Surface-release mechanisms. Following CnPlb1 export to raft membranes, some CnPlb1 is relocated to the cell wall and/or is secreted, though the mechanism of release is not known (Siafakas et al., 2006, 2007). The membrane GPI anchor is a potential substrate for (G)PI-PLC, (G)PI-PLD (de Almeida and Heise, 1993; Kristiansen and Richter, 2002) and/or a number of different proteases (Muller et al., 1996; Parkin et al., 2004; Gagnon-Arsenault et al., 2008) and/or α -mannosidase (Fujii et al., 1999; Kitagaki et al., 2002). We recently showed that secretion of CnPlb1 is dependent on *PLC1* which encodes a PI-PLC (Chayakulkeeree et al., 2008). However the mechanism by which *PLC1* achieves this is unclear (discussed in more detail below). Cell wall CnPlb1 associates with β -1,6-glucan (Siafakas et al., 2007) which, in *C. neoformans*, is much more abundant than β -1,3-glucan relative to other yeasts (Doering, 2009). β -1,6-glucans are also associated with secreted CnPlb1, suggesting that secreted CnPlb1 is derived from the cell wall reservoir (Siafakas et al., 2007). Disruption of specific *KRE* family genes in *C. neoformans* (*KRE5* and *KRE6/SKN1*), which in *S. cerevisiae* are required for synthesis of β -1,6-glucan, caused a reduction in the β -1,6-glucan content of the cell wall (Gilbert et al., 2010). This reduction in β -1,6-glucan coincided with compromised cell wall integrity and reduced retention of CnPlb1 in the cell wall (Gilbert et al., 2010) supporting the previous finding that CnPlb1 is retained in the cell wall via a linkage to β -1,6-glucan (Siafakas et al., 2007). That β -1,6-glucans are retained on secreted CnPlb1 suggests that extracellular endo β -glucanases may be involved in the release of CnPlb1 from the cell wall, in addition to fulfilling a traditional role in cell wall remodeling.

Extracellular glucanase activity, including α -mannosidase, α - and β -glucosidase, α - and β -galactosidase, β -xylosidase, β -glucuronidase, and endo β -1,3-glucanase is associated with the capsule and/or the cell wall of *C. neoformans* and some of the substrates of these enzymes can support yeast growth (Macekova et al., 2006). Synthesis of the majority of these enzymes is regulated by glucose repression (Macekova et al., 2006). It has been demonstrated that many cells die as they enter stationary phase due to erosion of their cell walls by autolysis, and that this coincides with an increase in externalization of β -1,3-glucanase activity with a pH

optimum of between 3.0 and 3.5 (Farkas et al., 2009). As stationary phase is also the time when CnPlb1 secretion is optimal, it is tempting to speculate that extracellular β -glucanases can release cell wall proteins, such as CnPlb1, into the external environment, while simultaneously making cell wall sugar available to nutrient-depleted cells in stationary phase. Six endo β -1,3-glucanase isoforms have been extracted from cryptococcal cell walls (Macekova et al., 2006). The *C. neoformans* genome encodes a family of four putative β -1,3-glucanases which could be involved in the release of CnPlb1. Cleavage of β -1,3-glucans, which in yeast are attached to β -1,6-glucans (Aimanianda et al., 2009), would result in the release of β -1,6-glucans and their attached proteins. Using gene deletion analysis, our laboratory is investigating the role of β -1,3-glucanases in CnPlb1 cell wall retention and secretion.

In addition to CnPlb1, the externalization of two other virulence determinants, laccase (Lac 1) and capsular glucuronoxylomannan (GXM), is dependent on fungal secretion pathways. Recent studies in *C. neoformans* defined separate pathways for export of Lac1 and GXM, which utilize *SEC6*-dependent exosomes of the lysosomal trafficking pathway and *SAV1(SEC4)*-dependent vesicles, respectively (Yoneda and Doering, 2006; Panepinto et al., 2009). Recently we determined that CnPlb1 cellular export and secretion in *C. neoformans*, but not that of Lac1 and GXM, is dependent on a gene homologs to *SEC14* from *S. cerevisiae*, which encodes a PI-transfer protein (PITP). This finding provides further support for the existence of distinct cellular pathways for export of virulence determinants in *C. neoformans* (unpublished data). Recently, several cryptococcal virulence determinants including laccase, urease, and soluble GXM polysaccharide, but not CnPlb1 to a significant extent, were shown to be transported across the cryptococcal cell wall to the external milieu via *SEC6*-dependent exosomes (Rodrigues et al., 2007, 2008; Casadevall et al., 2009; Panepinto et al., 2009). The mechanism of release of these virulence determinants from exosomes is unknown, but might involve the membrane hydrolytic action of cell wall-associated phospholipases such as CnPlb1.

ROLE OF PLB1 IN PATHOGENESIS

The relative importance of the three phospholipase B activities (PLB, LPL, and LPTA) in fungal pathogenesis may vary in the different host microenvironments. It has been shown that the LPL and LPTA activities of CnPlb1 can recylate lysophospholipids and reverse the damage caused to neutrophils by other secreted cryptococcal components (Santangelo et al., 1999). The roles of the various activities in pathogenesis is discussed in more detail below.

HOST ADHESION/PENETRATION AND DISSEMINATION

Plb enzymes are proven fungal invasions in *C. albicans* and *C. neoformans*. The first barriers encountered by infectious *C. neoformans* propagules are lung surfactant and lung epithelial cells. DPPC and PG, which are abundant in lung surfactant and dioleoyl PC (DOPC), which is present in the outer leaflet of mammalian cell membranes, are preferred substrates of CnPlb1 (Chen et al., 1997a, 2000). Hydrolysis of these phospholipids by CnPlb1 would potentially facilitate penetration of host tissue and establishment of cryptococcal infection. In support of this, partially purified CnPlb1 hydrolyses lung surfactant (Santangelo et al., 1999) and facilitates

adherence of cryptococci to lung epithelial cells (Ganendren et al., 2006). CnPlb1 also facilitated the entry of endotracheally administered cryptococci into lung interstitial macrophages although the mechanism of traversal across the lung epithelial barrier remains unknown. The reduced lung burdens of cryptococci observed after infection with the CnPlb1 deletion mutant (Δ plb1) may be explained by decreased invasive potential; it is also possible that multiplication within the host is inhibited. CnPlb1 is also essential for dissemination of *C. neoformans* from the lung to the brain via the blood, and possibly, the lymphatic system (Cox et al., 2001; Noverr et al., 2003; Santangelo et al., 2004). However, CnPlb1 is not essential for the establishment of neurological infections (Santangelo et al., 2004).

Cryptococcus neoformans is a facultative intracellular pathogen that survives and replicates within the intracellular phagolysosome of macrophages. Yeast–macrophage interactions result in several outcomes which potentially drive the establishment, progression, and dissemination of infection. Using a non-activated murine alveolar macrophage (AM) cell line, MHS-1, Noverr et al. (2003) showed that CnPlb1 is required for *C. neoformans* survival in the presence of these macrophages. Cox et al. (2001) showed that CnPlb1 promotes cryptococcal budding at early time points (1–3.5 h) post-phagocytosis by an activated J774 macrophage cell line. In contrast, phagocytosis of strain H99 ($7.3\% \pm 1.2$) by the phorbol-12-myristate 13-acetate-activated human macrophage-like cell line, THP-1, was significantly greater than that of Cn Δ plb1 ($4.3\% \pm 0.8$) (Wright et al., 2007). Not only does CnPlb1 facilitate entry of cryptococci into interstitial macrophages in the lung, but as demonstrated by Santangelo et al. (2004), it is also required for dissemination from the lungs to regional lymph nodes where cryptococci were observed to multiply within Langerhans-type giant cells (Santangelo et al., 2004). Higher brain burdens were observed following intravenous injection of lung macrophages infected with cryptococci compared with free cryptococci. Since the brain burden was independent of CnPlb1 it was concluded by the authors that macrophages provide a vehicle for dissemination of cryptococci to the brain (Santangelo et al., 2004). Further support for this was provided by Charlier et al. (2009).

CnPlb1 may also be required for cryptococci to exit macrophages and thereby avoid killing by macrophage immune defense mechanisms. Escape can occur either by macrophage lysis and cell death, or by non-lytic extrusion via a process that does not affect the viability of either *C. neoformans* or the host macrophage (vomocytosis) (Alvarez and Casadevall, 2006, 2007; Ma et al., 2006, 2007). The acidic environment of macrophage phagolysosomes provides an optimal environment for CnPlb1 catalytic activity. Feldmesser et al. (2000) reported disruptions in the phagolysosomal membrane of engulfed cryptococci which they attributed to secreted CnPlb1 and/or proteases. Such membrane disruptions may allow lysis-associated egress of *C. neoformans* from macrophages. Secreted CnPlb1 was recently found to facilitate non-lytic extrusion of *C. neoformans* from macrophages; two CnPlb1 strains defective in CnPlb1 secretion, due to *CnPLB1* deletion or deletion of a secretory factor required for normal secretion of CnPlb1, exhibited reduced rates of vomocytosis, and failure to disseminate to the brain in a mouse model (manuscript submitted).

Although the role of secreted CaPlb1 in adhesion of *C. albicans* to host epithelium is not well-established, there is evidence that CaPlb1 does play a role in damage to, and penetration of, host epithelium, as culture supernatants from wild-type *C. albicans* caused twice as much damage to epithelial cells as those from a *CaPLB1*-deficient strain (Ghannoum, 2000). Furthermore, CaPlb1 activity is concentrated at the growing tip of the invading hyphae (Mukherjee et al., 2001).

IMMUNOMODULATION

CnPlb1 is essential for the synthesis of bioactive anti-inflammatory eicosanoids by *C. neoformans*, both *in vitro* and *in vivo* (Noverr et al., 2002, 2003), and therefore has the potential to suppress the host immune system, promoting the survival, and dissemination of *C. neoformans* within the host. Arachidonic acid (AA) is the precursor of eicosanoids. *C. neoformans* lacks arachidonic acid (AA), but readily takes it up *in vitro* (Wright et al., 2007). Furthermore during phagocytosis, macrophage-derived AA can be incorporated into cryptococcal lipids by a process that is dependent on the LPTA activity of CnPlb1 (Wright et al., 2007). CnPlb1 is also essential for the synthesis of eicosanoids derived from diarachidonyl PC (Noverr et al., 2003). However, Wright et al. (2007) found evidence to suggest that free AA generated by the host, presumably by cPLA₂, is the substrate for CnPlb1-dependent eicosanoid production in *C. neoformans*, as neither wild type nor Cn Δ plb1 could release AA from the *sn*-2 position of the more physiologically relevant phospholipid, palmitoylarachidyl PC (PAPC). In *C. albicans*, the PLA₂ activity of CaPlb5 can release AA from PAPC (Theiss et al., 2006). This is consistent with one role for CaPlb5 in pathogenesis, being to generate bioactive lipid mediators, such as AA, from host membranes. This AA potentially provides precursors for pro- or anti-inflammatory compounds including eicosanoids (Theiss et al., 2006). This process may also coincide with the breakdown of host cell membranes leading to cell lysis.

FUNGAL NUTRITION, MEMBRANE REMODELING, SIGNALING AND CELL WALL INTEGRITY

Cryptococcus neoformans can utilize both phospholipids and fatty acids as a sole carbon source, and CnPlb1 is required for the uptake and esterification of fatty acids during stress (Wright et al., 2007). Thus during infection, CnPlb1-dependent hydrolysis of phospholipids in host membranes could provide an energy source and confer a survival advantage on *C. neoformans* within the low glucose environments of the macrophage phagolysosome and cryptococcomas, particularly as CnPlb1 enzymatic activity is energy-independent (Wright et al., 2007). Consistent with this hypothesis, Cn(Δ plb1) has a slower rate of intracellular growth than the wild-type strain at early time points post-phagocytosis (Cox et al., 2001). Wright et al. (2007) also demonstrated CnPlb1-dependence of metabolism of the physiologically relevant lipids, DPPC and DOPC, and the cellulolytic compound lysoPC under stress conditions, and of detoxification of lysoPC by LPTA.

The CnPlb1-derived product of phosphatidylcholine, GPC, which is produced within cryptococcomas, *in vivo*, could be derived from either the yeast or the host. However we have reported that GPC is present in supernatants of cryptococci cultured *in vitro* (Bubb et al., 1999). As the secretion medium lacked phospholipid and CnPlb1 is

the only enzyme with PLB activity secreted under these conditions, the GPC must have been derived from cryptococcal lipid by the hydrolytic action of CnPlb1. Localization of CnPlb1 in the outer leaflet of membranes with lipid raft-like properties, provides circumstantial evidence for a role within membranes (Siafakas et al., 2006). Interestingly however, PLB activity is, in fact, suppressed in the raft environment (Siafakas et al., 2006) suggesting that sequestration of CnPlb1 in lipid rafts may be a mechanism to prevent fungal membrane damage and/or regulate lipid remodeling within the outer membrane leaflet. Presumably any membrane homeostatic effect of CnPlb1 would occur only after release from the GPI anchor. Although lipid rafts generally facilitate signal activation, there is precedent for suppression of enzyme activity within the raft environment, in this case for a metalloproteinase (Kim and Chung, 2008).

It is also plausible that CnPlb1 is involved in fungal cell signaling following hydrolysis of the GPI anchor, which simultaneously releases CnPlb1 from raft membranes and generates the signaling molecule, diacylglycerol (DAG). In support of this, GPI anchored proteins are thought to be involved in signal transduction in mammalian cells (Stulnig et al., 1997). CnPlb1 could also regulate lysophosphatidic acid (LPA) levels which, in mammalian cells, are associated with regulation of PKC (Holmstrom et al., 2010). CnPlb1 also contributes to cell wall integrity (Chayakulkeeree et al., 2008), either by the physical nature of its GPI-anchored status in the cell wall, by maintaining the integrity of the membrane to which the cell wall is fixed or via a role in cell wall-related signal transduction involving PKC. In fungi, PKC is an enzyme within the PKC/Mpk1 cell wall integrity pathway, but is also a central regulator of signal transduction pathways that impacts on host cell physiology (Gerik et al., 2008).

MOLECULAR MODELING AND SITE-DIRECTED MUTAGENESIS REVEAL A SINGLE ACTIVE SITE IN CNPLB1

Previous studies demonstrated that several agents, including a CnPlb1 peptide-directed antibody, differentially inhibit either the LPL/LPTA, or the PLB activities of secreted CnPlb1 (Chen et al., 2000; Ganendren et al., 2004), suggesting that CnPlb1 has more than one active site. However, site-directed mutagenesis and molecular modeling in CnPlb1 (Jones et al., 2007) based on the X-ray crystal structure of the catalytic domain of mammalian cytosolic phospholipase A₂ (cPLA₂) (Dessen, 2000), revealed that CnPlb1 has only a single active site consisting of the same catalytic residues (S146, D392, and R108) as those involved in catalysis of nearly all fungal PLBs (Pickard et al., 1996) and human cPLA₂ (S228, D549, and R200) (Jones et al., 2007). Comparison of the CnPlb1 molecular model and the cPLA₂ crystal structure revealed the presence of a hydrophobic binding pocket, which accommodated the catalytic residues in identical positions, with S/D forming a catalytic dyad (Jones et al., 2007). In both structures, R108/R200 is sufficiently distanced from the S/D catalytic dyad to stabilize binding of the phospholipid head group and to allow cleavage of the acyl chain of the substrate, by the catalytic dyad (Jones et al., 2007). *In silico* substrate docking studies revealed that each phospholipid acyl chain is accommodated by an upper and a lower hydrophobic binding tract and that fatty acyl cleavage at the *sn*-2 ester bond occurs within the upper binding tract. Sequence hypervariability was identified in the CnPlb1 and cPLA₂ upper binding tracts, providing a potential

explanation for why cPLA₂ has a preference for AA in the *sn*-2 position and removes only this fatty acid (Dessen, 2000), whereas CnPlb1 prefers either palmitoyl or oleoyl fatty acid in both the *sn*-1 and *sn*-2 positions and can remove both via the PLB activity (Chen et al., 2000) but cannot remove AA from the *sn*-2 position (Wright et al., 2007).

Sequence hypervariability was also identified in the binding tracts of the CnPlb1 active site providing a potential explanation for why various inhibitors differentially affect the three CnPlb1 enzyme activities. This could occur if the different inhibitors exhibited a preference for interaction with one binding tract over another. Alternatively, the initial point of contact of the substrate with the enzyme may not be the active site and may differ for phospholipids and lysophospholipids. Molecular modeling also revealed the absence of a "lid" within the CnPlb1 cap domain which, in cPLA₂, occludes the active site and is involved in interfacial activation. However, since CnPlb1 also displays interfacial activation in the absence of a lid, an alternative explanation is that this region is involved in substrate specificity, consistent with hypervariability in this region among cPLA₂ isoforms and between cPLA₂ and CnPlb1. Hence the lid region may function to recognize the appropriate lipid substrate in a membrane.

FUNGAL PLBS AS ANTIFUNGAL DRUG TARGETS

In light of the paucity of antifungal drug classes, the increasing incidence of relapsing invasive fungal infections and primary drug resistance, and unfavorable toxicity/bioavailability profiles of currently marketed antifungals, investigation of alternative compounds as stand-alone or synergistic therapies, is urgently needed. Fungal Plb and/or its biosynthetic pathway are novel targets for drug development and compounds with structural similarities to LysoPC, which is a preferred substrate of CnPlb1, have already been assessed for their antifungal effect. For many, there was a correlation between antifungal and anti-CnPlb1 activity but not all demonstrated selectivity for CnPlb1 compared with mammalian phospholipase, and some were toxic to mammalian cells.

Early studies using four commercially available compounds [alexidine dihydrochloride (compound A), dioctadecyldimethylammonium bromide (compound O), 1,12 bis-(tributylphosphonium)-dodecane dibromide (compound P), and decamethonium dibromide (compound D)], showed both selectivity for CnPlb1 and antifungal activity. The best CnPlb1 inhibitors (compounds A and P) displayed the most potent antifungal activity. Compound A was highly selective for secreted and cell-associated PLB activities and showed no inhibition of mammalian PLA₂. Compound O, which was specific for secretory and cytosolic LPL and LPTA activities and membrane-associated PLB activity, was not antifungal (Ganendren et al., 2004). Non-specific detergent-like action of the compounds was ruled out by the fact that they exhibited a selective inhibitory effect on the different enzyme activities.

These initial observations prompted us to synthesize and test three classes of compound with structural similarities to lysoPC, as potential antifungal agents. Alkyl-bis(phosphonium) analogs of these were active against yeasts and filamentous fungi including *A. fumigatus*, *A. terreus*, and *Scedosporium apiospermum*, with MICs similar to those of Amphotericin B (Obando et al., 2007). Although

promising, the selectivity of these prototype compounds for CnPlb1 was unsatisfactory. In contrast, the most promising compound of a novel structural class of bis-pyridinium compounds, was selectively active against CnPlb1, non-toxic to human erythrocytes and nucleated mammalian cells and exhibited broad-spectrum antifungal activity (Obando et al., 2009). This compound is currently being tested for toxicity in animals.

Our search for compounds with substrate similarities to phospholipids yielded an additional candidate drug, miltefosine (hexadecylphosphocholine), which has been marketed as an oral formulation for the treatment of leishmaniasis. Miltefosine is a metabolically stable lysophospholipid analog which we reported to have broad-spectrum fungicidal activity *in vitro* against yeasts and molds, including resistant species such as *Candida krusei*, *C. glabrata*, *S. prolificans*, *Fusarium* species, and *Zygomycetes* (Widmer et al., 2006). MICs are well below serum levels achieved by oral dosing with miltefosine and there is very preliminary evidence of an effect in a mouse model of cryptococcosis and as combination salvage therapy in a child with severe, non-responsive, *S. prolificans* infection (Kesson et al., 2009). Because of its undesirable gastrointestinal side-effects, the antifungal mode(s) of action of miltefosine are under investigation as a prelude to the development of more potent, less toxic analogs. To date, we have established that miltefosine does inhibit CnPlb1, but only at high concentrations, suggesting that this is not its major mode of action (Widmer et al., 2006). Analogs of miltefosine have been synthesized and are under investigation.

In conclusion, although the mature CnPlb1 enzyme is a potential target for antifungal drug development, inhibitor compounds investigated so far appear to exert their antifungal effect through additional pathways. An alternative approach under active investigation is to seek to inhibit cellular synthesis and/or secretion of CnPlb1 (Siafakas et al., 2007).

ROLE OF FUNGAL PHOSPHOLIPASE C ENZYMES IN FUNGAL FITNESS AND PATHOGENICITY

HOW FUNGAL PLCs DIFFER FROM THE MAMMALIAN HOMOLOGS

The *C. neoformans* genome contains two genes, *PLC1* and *PLC2*, encoding putative phospholipase C enzymes (Chayakulkeeree et al., 2008). *CnPLC1* encodes a protein that is predicted to be 609 amino acids (CnPlc1), with X and Y catalytic domains that are characteristic of eukaryotic Plcs, including the homologs from *C. albicans* (CaPlc1) (Kunze et al., 2005) and *S. cerevisiae* (ScPlc1) (Flick and Thorner, 1993; Yoko-o et al., 1993). Like CaPlc1 and ScPlc1, CnPlc1 contains a C2 domain which is predicted to bind Ca²⁺, and is 26% similar at the amino acid level to both proteins (Chayakulkeeree et al., 2008). In contrast to ScPlc1 and the mammalian Plc- δ homolog which is most similar (32%) to the fungal homologs, CnPlc1 and CaPlc1 lack the EF hand regulatory domain suggesting differences in their mechanism of action. Furthermore, all of the fungal Plcs, lack the pleckstrin homology (PH) membrane binding domain which is present in mammalian Plc- δ . Other potentially significant differences between the fungal and mammalian enzymes include the presence of an unusually long N-terminal extension in the fungal enzymes (Flick and Thorner, 1993; Bennett et al., 1998), and observation that fungal enzymes are predicted to be basic while the rat and bovine enzymes are acidic (Flick and Thorner, 1993). These

differences, combined with the fact that deletion of fungal *PLC1* genes leads to severely compromised growth phenotypes (Flick and Thorner, 1993; Payne and Fitzgerald-Hayes, 1993; Yoko-o et al., 1993; Chayakulkeeree et al., 2008), or lethality in the case of *C. albicans* (Kunze et al., 2005), highlights the potential of Plc enzymes as antifungal drug targets. In contrast to *C. neoformans* and *C. albicans*, *ScPLC1* is the sole Plc in *S. cerevisiae*.

CnPLC2 encodes a 430 amino acid protein which contains an X catalytic domain but no C2 domain. It is therefore more similar to prokaryotic PI-PLCs, many of which are proven pathogenicity determinants (Poussin and Goldfine, 2005; Poussin et al., 2009). Although not essential for pathogenicity in *C. neoformans*, CnPlc2 may provide some functional redundancy as deletion of *CnPLC1* is not lethal (Chayakulkeeree et al., 2008). In contrast, deletion of *CaPLC1* is lethal in *C. albicans*, despite the presence of two prokaryote-like Plc-encoding genes, *CaPLC2* and *CaPLC3*, which therefore cannot functionally compensate for loss of *CaPLC1* (Kunze et al., 2005). *CaPlc2* and *CaPlc3* both share homology to PlcA (a PI-PLC), which is a known virulence factor of the human pathogen, *Listeria monocytogenes* (Wei et al., 2005). However in contrast to *L. monocytogenes*, *CaPlc2* and *CaPlc3* are dispensable for virulence and yeast-macrophage interactions. Additionally, they are not required for yeast-hyphal switching, despite augmented mRNA expression under hypha-inducing conditions (Knechtle et al., 2005). The function of CnPlc2, *CaPlc2*, and *CaPlc3* remains to be elucidated.

PLC – ROLE IN FUNGAL PATHOGENICITY

Plcs hydrolyse the membrane-proximal phosphoester bond of the phosphoryl head group of PI phospholipids and/or their phosphorylated and glycosylated derivatives [PI(4)P/PI(4,5)P/PIP₂ and (G)PI, respectively]. The eukaryote-type enzymes prefer PIP₂ as a substrate, over PI, while the prokaryote types prefer PI. Plcs from bacteria and some protozoan parasites are more efficient at cleaving GPI anchors and are therefore sometimes referred to as (G)PI-PLCs (Heinz et al., 1998). The release of CnPlb1 from membranes in *C. neoformans*, which allows its translocation to the cell wall and/or the extracellular environment, may be instigated by (G)PI-PLC activity. Surprisingly though, deletion of the more prokaryote-like *CnPLC2* in *C. neoformans*, did not alter CnPlb1 secretion and was dispensable for phenotypic expression of pathogenic traits and virulence in an animal model (Chayakulkeeree et al., 2008), consistent with any potential CnPlc2 functionality being fully compensated for by CnPlc1. In contrast, deletion of the more eukaryote-like *CnPLC1* (*Cn Δ plc1*), abrogated Plb1 secretion in broth cultures of *C. neoformans* (Chayakulkeeree et al., 2008). As CnPlc1 is not predicted to be secreted, and would therefore not have access to extracellular GPI anchors, the CnPlb1 secretion block in *Cn Δ plc1* should not be due to compromised GPI anchor cleavage in *Cn Δ plc1*. Attempts to make a *CnPLC1/PLC2* double deletion mutant were unsuccessful suggesting that loss of these two genes is lethal and that CnPlc2 is functional even though it cannot fully compensate for the loss of CnPlc1 (Chayakulkeeree et al., 2008).

CnPLC1 is essential for a number of other key survival and pathogenic determinants, including the ability of *C. neoformans* to grow at physiological temperature; which predictably leads to avirulence of *Cn Δ plc1* in a mouse infection model (Chayakulkeeree et al., 2008). Even in a *Caenorhabditis elegans*

killing assay performed at the permissible growth temperature of 28°C, Cn Δ plc1 was less virulent than WT, demonstrating an effect not solely explained by the 37°C growth defect of this mutant (Chayakulkeeree et al., 2008). Furthermore, Cn Δ plc1 could not make the stress-protecting pigment, melanin, due to suppression of transcription of the laccase-encoding gene, *LAC1*, and displayed a cell morphology defect, compromised cell wall integrity and hypersusceptibility to clinically available antifungal drugs (Chayakulkeeree et al., 2008). Recently, we also demonstrated that capsules cannot be induced in Cn Δ plc1 (Unpublished observation). Thus in contrast to *C. albicans*, *C. neoformans* can compensate for loss of *PLC1* at temperatures of $\leq 30^\circ\text{C}$. This is most likely achieved through functional adaptation by CnPlc2 which may account for the residual Plc activity in Cn Δ plc1 cell lysates (Chayakulkeeree et al., 2008).

PLC – ROLE IN OTHER FUNGI

PLC1 orthologs in other fungi appear to have a similar function to Cn Δ PLC1. However, unlike in *C. neoformans* and *S. cerevisiae*, Ca Δ PLC1 is an essential gene, as attempts to disrupt both Ca Δ PLC1 alleles failed and disruption of the second allele was only possible in mutants with Ca Δ PLC1 gene duplication (Kunze et al., 2005). Furthermore, growth of *C. albicans* was blocked in the presence of the Plc-specific inhibitor, ET-18 (Kunze et al., 2005). A conditional Ca Δ PLC1 mutant had phenotypes similar to the *PLC1* deletion mutant in *S. cerevisiae* (Flick and Thorner, 1993; Payne and Fitzgerald-Hayes, 1993) and *C. neoformans* (Chayakulkeeree et al., 2008), including sensitivity to osmotic stress, elevated temperature and growth on carbon sources other than glucose (Kunze et al., 2005). Additionally, this conditional mutant had defects in hyphal formation (Kunze et al., 2005). Other defects apparent in the Sc Δ PLC1 deletion mutant are in morphology/budding and chromosome segregation (Payne and Fitzgerald-Hayes, 1993).

PHOSPHOLIPASES – A ROLE IN NON-FUNGAL PATHOGENS

Phospholipases are virulence determinants of bacteria and protozoa, as well as fungi, and some examples are given below. *Rickettsia rickettsii*, the causative agent of life-threatening Rocky Mountain spotted fever, is an obligate intracellular bacterium. Phospholipase A₂ is involved in rickettsial entry into host cells, escape from macrophage phagosomes and host cell lysis (Silverman et al., 1992). Phospholipase A also facilitates dissemination from the host cell by two protozoan species, *Toxoplasma gondii* and *Entamoeba histolytica* (Ghannoum, 2000). Recently, a protein with phospholipase A activity was identified from *R. typhi* (Rahman et al., 2010).

PI-PLC from *L. monocytogenes* promotes escape of the bacterium from macrophage phagolysosomes via a mechanism that is dependent on host Pkc β , which may be activated by PI-PLC-generated DAG (Poussin and Goldfine, 2005; Poussin et al., 2009). The broad-range phospholipase C, PC-PLC, is also involved in vacuolar lysis by *L. monocytogenes*, allowing bacterial growth within the host cell cytosol, and spread from cell to cell via actin-based motility. Recently, it was shown that slowing the kinetics of vacuolar maturation in *L. monocytogenes* creates an environment conducive for PC-PLC-assisted vacuolar escape (Burrack et al., 2009). In another bacterium, *Clostridium perfringens*, Plc causes gas gangrene (Kennedy et al., 2009).

CNPLC1 – A POTENTIAL BRANCH POINT ENZYME FOR INTEGRATING VIRULENCE-RELATED SIGNAL TRANSDUCTION

There are at least six signaling pathways in *C. neoformans* that regulate virulence (reviewed in Kozubowski et al., 2009). The CnPlc1-dependent virulence phenotypes (melanin, capsule, high temperature growth, and cell wall integrity) are regulated by three of these: the cAMP/protein kinase A (Pka) pathway, Ca²⁺/Calcineurin pathway and the protein kinase C1 (Pkc1)/mitogen activated protein kinase 1 (Mpk1) pathway. High temperature growth and cell wall integrity are regulated through the Ca²⁺/Calcineurin and Pkc1/Mpk1 pathway. There is also evidence of cross-talk between the Ca²⁺/calcineurin and the Pkc1/Mpk1 pathways to regulate cell wall integrity in *C. neoformans*. Firstly, the mRNA of CnFKS1, which is a gene target of the Pkc1/Mpk1 pathway, is upregulated by loss of calcineurin function (Kraus et al., 2003). Secondly, inhibition of calcineurin by FK506 induces CnFKS1 mRNA expression and activates CnMpk1 (Kraus et al., 2003). Finally, melanin, capsule, and mating are regulated through the cAMP/Pka pathway (Alspaugh et al., 1997; Alspaugh et al., 2002). Whether production and/or secretion of the invasin, CnPlb1, is also regulated by one of these signaling pathways, is yet to be determined.

Hydrolysis of PIP₂ and, to a lesser extent of PI, was reduced in Cn Δ plc1 (Chayakulkeeree et al., 2008), providing evidence that CnPlc1 is a functional enzyme involved in signaling. Preferential hydrolysis of PIP₂ over PI generates two intracellular secondary messengers, 1,2-diacylglycerol (DAG) (the sole hydrolysis product of PI) and inositol 1,4,5-trisphosphate (IP₃) which can activate the Pkc1/Mpk1 and Ca²⁺/Calcineurin pathways, respectively. It has been shown that DAG generated by the sphingolipid biosynthetic pathway activates CnPkc1 to regulate cell wall integrity and melanin production (Heung et al., 2005). Thus, CnPlc1-generated DAG could play a similar role. In support of this, Cn Δ plc1 has a cell wall integrity defect and Mpk1 is not activated in Cn Δ plc1 following cell wall perturbation (Chayakulkeeree et al., 2008). Thus, CnPlc1 can potentially integrate signaling through both the Pkc1/Mpk1 and Ca²⁺/Calcineurin pathways to regulate high temperature growth and cell wall integrity, and potentially, melanin production (see **Figure 3**). CnPlc1 signal transduction may be influenced by PI-3 kinase, which is a potential competitor for PIP₂ substrate and regulator of melanization and autophagy virulence traits in *C. neoformans* (Hu et al., 2008), and the phospholipid-binding protein, Cts1, which functions coordinately with calcineurin in *C. neoformans* to regulate growth and cell separation (Fox et al., 2003).

In *S. cerevisiae*, there is evidence that ScPlc1 signals through cAMP-regulated pathways. Firstly, ScPlc1 modulates interaction of the putative nutrient sensing G-protein coupled receptor, Gpr1p, with the G α protein, Gpa2p, effecting cAMP-regulated filamentation (Ansari et al., 1999). Secondly, cAMP synthesis is decreased in Sc Δ plc1 (Demczuk et al., 2008). In *C. neoformans*, a protein complex of Gpr4p, Gpa1p, and Crg2p, functions to control cAMP signaling via Pka (Kozubowski et al., 2009) to regulate melanin and capsule production, and mating. Support for CnPlc1 being linked to this signaling pathway is that Cn Δ plc1 has a melanin defect, as a result of reduced *LAC1* mRNA transcription, and a capsule defect (Chayakulkeeree et al., 2008) and unpublished data, respectively (see **Figure 3**). A comparative transcriptomic study of a *C. neoformans* Pka catalytic subunit deletion mutant, and the WT, identified a role for the cAMP/Pka pathway in secretion and, in particular, secretion

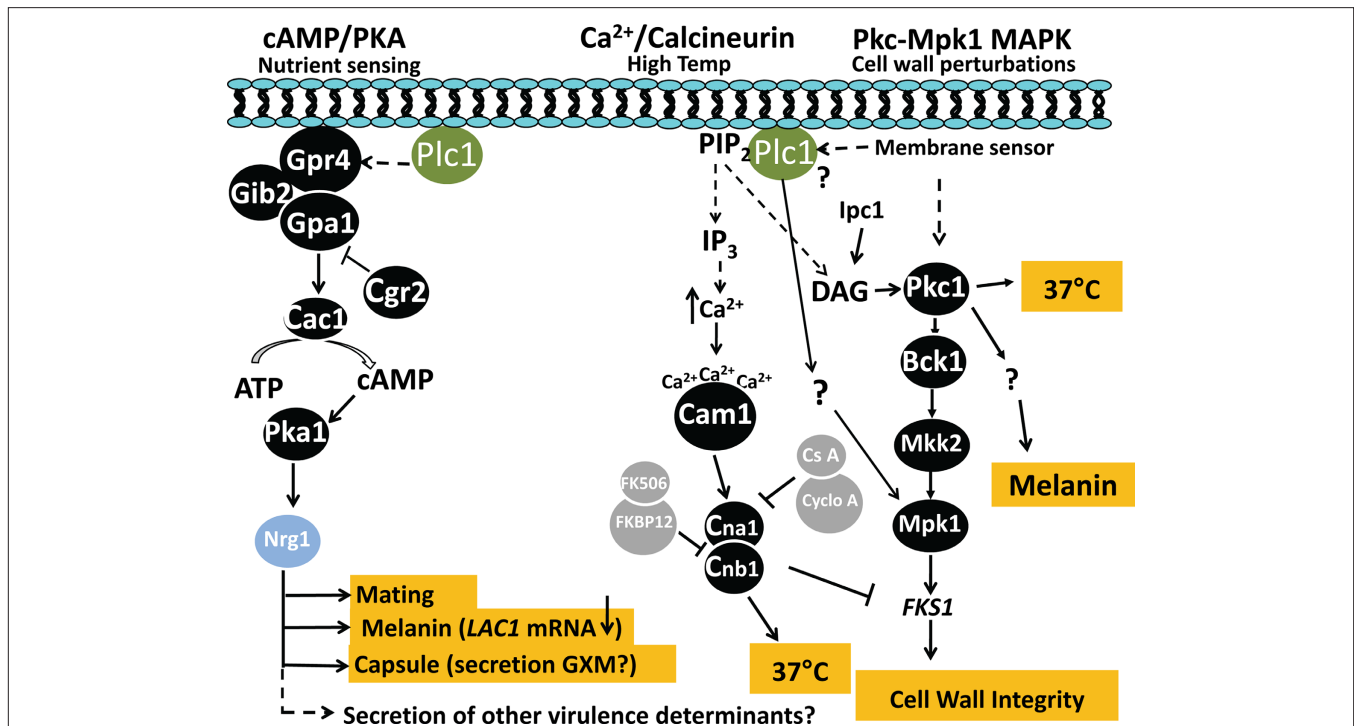


FIGURE 3 | Proposed role of CnPlc1 in pathogenicity-related signaling in *C. neoformans*. 1. Cyclic AMP (cAMP)/Protein kinase A (Pka) signaling pathway. A change in extracellular nutrient levels trigger activation of the integral membrane-associated G-protein coupled receptor, GPR4, causing its associated G α subunit (Gpa1) to activate adenylyl cyclase (Cac1), resulting in production of cAMP. cAMP binds to regulatory subunits (Pkr1) of the Pka complex (not shown) to release an active form of the catalytic subunit (Pka1), which phosphorylates the transcription factor, Nrg1. A GTPase-activating protein (Crg2) negatively regulates Gpa1. Gib2, G β subunit. Based on studies in *S. cerevisiae* and our own published and preliminary data in *C. neoformans* demonstrating the requirement for CnPlc1 in melanin production (via *LAC1* transcription), capsule production and secretion of virulence determinants (Chayakulkeeree et al., 2008), CnPlc1 may be linked to cAMP signaling possibly by regulating the activation of Gpa1 as indicated by the dashed line. Lac1, Laccase 1; GXM, glucuronic acid-xylose-mannan. 2. Ca²⁺/calcineurin signaling pathway. This pathway primarily regulates high temperature growth in *C. neoformans*, but not in *S. cerevisiae*. High temperatures may trigger CnPlc1 to hydrolyse PIP₂, producing IP₃, which potentially raises intracellular Ca²⁺ (see dashed line) by regulating Ca²⁺ pumps/channels in the plasma membrane

and ER. Cytosolic Ca²⁺ is sensed by calmodulin (Cam1), which activates calmodulin kinases (CaMKs) (not shown) and the calcineurin complex (Cna1 and Cnb1) to support growth at 37°C. Cyclosporine A (CsA) and FK506, bind cyclophilin A and FKBP12, respectively, to inhibit calcineurin; PIP₂, phosphatidylinositol 4,5-bisphosphate. Calcineurin is also essential for hyphal elongation during mating and monokaryotic fruiting (not shown). 3. Pkc1/Mpk1 MAPK signaling pathway. This pathway conveys cell wall perturbation signals detected by unknown membrane sensors, to the nucleus. CnPlc1 is essential for activation of Mpk1 in response to cell wall perturbation in a process that may, or may not be, mediated by activation of Pkc1 via the CnPlc1 hydrolysis product, DAG (see dashed line). Activated Pkc1 phosphorylates MAPKKK (Bck1) and subsequent phosphorylations of MAPKK (Mkk2) by Bck1 and MAPK (Mpk1) by Mkk2 to upregulate transcription of *FKS1*, which encodes β -1,3-glucan synthase. Expression of *FKS1* is also negatively regulated by calcineurin. DAG produced by the sphingolipid synthetic pathway enzyme inositolphosphoceramide 1 synthase (*Ipc1*), and possibly by CnPlc1, also activates CnPlc1 to regulate melanin production independently of Mpk1 activation (Heung et al., 2005). Pkc1 is also required for host temperature growth (Gerik et al., 2008).

of capsular polysaccharide (Hu et al., 2007). However, secretion of protein-based virulence factors was not assessed. Further support for CnPlc1 being linked to the cAMP/Pka pathway in *C. neoformans*, is that CnPlb1 secretion is defective in *Cn Δ plc1* (Chayakulkeeree et al., 2008). A comparison of secreted CnPlb1 in Pka mutants and WT is currently under investigation to determine whether this pathway does, in fact, play a role in controlling the secretion of pathogenicity determinants other than capsular polysaccharide.

In summary, CnPlc1 may represent an important branchpoint signaling enzyme and its relative contribution to individual signaling pathways will be determined through epistasis studies. These studies

will confirm whether or not three individual signaling pathways constitute a regulatory network in which CnPlc1 plays a central role. The elucidation of CnPlc1 function within the model fungal pathogen, *C. neoformans*, will therefore fill some essential gaps within our understanding of signaling networks of pathogenic fungi, which can differ substantially from those of *S. cerevisiae*, and invites investigation of similar pathways in other pathogenic fungi.

ACKNOWLEDGMENTS

The author would like to thank Mr Stephen Schibeci and Professor Tania Sorrell for proof-reading this review.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 August 2010; accepted: 25 October 2010; published online: 11 November 2010.

Citation: Djordjevic JT (2010) Role of phospholipases in fungal fitness, pathogenicity, and drug development – lessons from *Cryptococcus neoformans*. *Front. Microbio.* 1:125. doi: 10.3389/fmicb.2010.00125
This article was submitted to *Frontiers in Fungi and Their Interactions, a specialty of Frontiers in Microbiology*.
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