Fostering Fungal Fitness and Pathogenicity - the Role of Phospholipases

Julianne Djordjevic*

Centre for Infectious Diseases and Microbiology, ICPMR and Westmead Millennium Institute, University of Sydney at Westmead Hospital, NSW 2145

**Corresponding author: julie_djordjevic@wmi.usyd.edu.au*

Cryptococcus neoformans – a Model for Elucidating Mechanisms of Fungal Pathogenicity

Opportunistic invasive fungal infections are increasing in prevalence, largely due to the continuing worldwide increase in immunocompromised individuals, especially in the developing world. The predominantly AIDS-related infection by the basidiomycetous fungal pathogen, *Cryptococcus neoformans*, is responsible for an estimated one million cases of meningitis and 675,000 deaths per year in HIV-infected patients alone (1). Furthermore, infections of immunocompetent individuals have also been reported (2). Although predominantly occupying a saprophytic environmental niche in soil, decaying material of trees including eucalypts and nitrogen-rich avian guano, *C. neoformans* is believed to have become acclimatised to infecting mammalian hosts as a result of phagocytic interactions with soil amoeba. Human macrophages phagocytose inhaled spores or small dessicated yeast cells, permitting a latent lung-localised infection. *C. neoformans* is, in fact, a facultative intracellular pathogen and in the event of immune deficit, can disseminate to other organs, with a predilection for the central nervous system and the establishment of severe life-threatening meningoencephalitis, which can be fatal if not treated.

In addition to being an important pathogen in its own right, *C. neoformans* is also recognised as an excellent model for studying fungal pathogenic mechanisms as its recently sequenced haploid genome is amenable to a range of genetic manipulation techniques, including targeted gene disruption, while robust animal infection models are available to assess the role of gene(s) in pathogenicity. Some of the most well-studied pathogenic determinants in *C. neoformans* include laccase-induced production of the stress-protecting pigment, melanin, production of urease which hydrolyses the urea found in avian habitats to ammonia, elaboration of a polysaccharide capsule to prevent yeast dessication and modulate host immune responses, and secretion of phospholipase B1 (Plb1). Plb is also a pathogenic determinant of other invasive fungal pathogens such as *Candida albicans* (3), and is produced by *Aspergillus* species. Although Plb1 is also secreted by non-pathogenic yeast, such as *Saccharomyces cerevisiae*, making the technical reference to Plb1 as a pathogenicity/virulence determinant inaccurate according to the refined Koch's molecular postulates of virulence (4), its role in pathogenicity, as shown in virulence models, is most likely attributed to the significantly higher specific activity of the secreted cryptococcal enzyme, compared with that of its *S. cerevisiae* orthologue (5). The secretion of Plb1 into the tissues of infected individuals has been demonstrated for both *C. neoformans* and *C. albicans*, supporting evidence for a role in invasion (6).

Phospholipase B1 – Role in *C. neoformans* Infection

Phospholipases are a heterogeneous group of enzymes that hydrolyse one or more ester linkages in glycerophospholipids. Cryptococcal Plb1 comprises phospholipase B, lysophospholipase and lysophospholipase transacylase activities, lending it the capacity to remodel cell membranes, derive energy from the fatty acid hydrolysis products, induce lysis of cell membranes and lung surfactant (both of which are rich in its preferred phosphatidylcholine substrate) facilitating fungal invasion and dissemination, and release immunomodulatory lipid signaling molecules. Targeted disruption of the *PLB1* gene leads to the abolition of secretion of all three Plb1 activities, attenuated virulence of *C. neoformans* (7) and altered immune responses *in vivo* (8). As Plb1 is a mannoprotein tethered to the fungal plasma membrane and cell wall by a glycosylphosphatidylinositol (GPI) anchor, it is not surprising that a cell wall integrity defect results from its absence (9). As antibodies to Plb1 have been detected in serum of patients infected with *C. neoformans* (10), Plb1, like other GPI-anchored mannoproteins, is a potential cryptococcal antigen capable of stimulating T cell responses. Attenuated virulence in the Δ*plb1* mutant strain coincided with reduced dissemination to the brain (7), a process which may be facilitated by macrophages that have phagocytosed the yeast and sequestered them inside phagolysosomes. Plb1 may play a role in allowing *C. neoformans* to survive within, and escape from, a phagolysosome, as the acidic environment of this subcellular compartment exacerbates the secretion of Plb1 and is conducive to optimal enzyme activation. Secreted Plb1 can therefore potentially allow the internalised yeast to derive energy from the fatty acids released from phagolysosomal membranes. In support of this, Feldmesser *et al.* (11) reported disruptions in the phagolysosomal membrane of engulfed cryptococci, which they attributed to secreted Plb1 and/or proteases. Digestion of the phagolysosomal membrane would also facilitate egress of *C. neoformans* from the macrophage.

Many cryptococcal pathogenicity determinants, including melanin-producing laccase, urease, the capsular polysaccharide building block, glucuronic acid-xylose-mannose (GXM), Plb1 and other GPI-anchored determinants of cell wall integrity, are all dependent on fungal secretion pathways for export to the cell periphery. Plb1 is most likely secreted by Golgi-derived vesicles,

rather than by exosomes of the lysosomal pathway, which are responsible for secretion of other virulence factors (12). We recently discovered that SEC14, which encodes a putative Golgi-localised phosphatidylinositol (PI)-transfer protein, is a requirement for secretion of Plb1 in *C. neoformans*, but not for capsule production and melanisation (unpublished data), providing further evidence that pathogenic determinants are exported to the cell periphery by more than one route.

Phospholipase C1 – an Essential Requirement for Plb1 Secretion and So Much More

Secretion of Plb1 and the translocation of similarly GPI-anchored proteins essential for cell wall integrity (including Plb1 itself) require release of the proteins from their membrane-embedded GPI-anchor. This process can be instigated by PI-specific phospholipase C enzymes (PLCs), which 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 /PIP_2 and (G)PI, respectively]. (G)PI-PLCs from bacteria and some protozoan parasites are the most efficient at cleaving GPI anchors.

The *C. neoformans* genome encodes a eukaryote-like PLC (*PLC1*) containing an X and Y catalytic domain and a prokaryote-like PLC (*PLC2*) containing only an X catalytic domain. Surprisingly, deletion of *PLC2* does not alter Plb1 secretion and it is dispensable for viability, expression of pathogenic traits and virulence (13), suggesting that potential Plc2 functionality is fully compensated for by Plc1 (13). In contrast, deletion of *PLC1* drastically abrogated Plb1 secretion (13). As Plc1 is not predicted to be secreted, and would therefore not have access to the extracellular GPI anchor, the Plb1 secretion block in the *PLC1* deletion mutant (Δ*plc1*) is not likely to occur as a consequence of the absence of a GPI anchor cleavage mechanism provided by Plc1. In fact, PLC1 is required to regulate a number of key survival and pathogenic determinants, including the ability to adapt to growth at mammalian host temperature, which predictably led to avirulence of Δ*plc1* in a mouse infection model (13). Growth of wildtype *C. neoformans* was inhibited to a greater extent at 37°C than at 30°C by the Plc inhibitor, U73122 (≥2.5 μM), mimicking the temperature-sensitive Δ*plc1* phenotype (13).

Transcription of the laccase-encoding gene, *LAC1*, was also suppressed in Δ*plc1*, leading to an albino phenotype in growth medium supplemented with the L-dopa precursor of the stress-protecting pigment, melanin (13). Δ*plc1* also had a clumping phenotype due to the inability to complete bud separation during cytokinesis, and could not regulate cell wall integrity in the presence of a range of cell wall perturbing agents. Potentially diminished secretion of GPI-anchored proteins in addition to that of Plb1 may have contributed to the cell wall defect. Interestingly, capsule production, and therefore the transport of GXM to the cell periphery, was not affected by loss of *PLC1*, providing further evidence of the existence of more than one route of secretion of pathogenic determinants in *C. neoformans*.

In contrast to other pathogenic yeast, such as *C. albicans* where *PLC1* is essential (14), *PLC1* deletion in *C. neoformans* is not lethal, demonstrating that *C. neoformans* has evolved mechanisms to compensate for loss of *PLC1* at temperatures of ≤30°C. This may be due to functional compensation by Plc2 which may account for the residual PLC activity in Δ*plc1* cell lysates (13). *C. neoformans* is therefore the only pathogenic fungal model available for investigating the role of *PLC1* in pathogenicity. Loss of *PLC1* also increased susceptibility of *C. neoformans* to antifungal drugs (13).

PLC1 and Pathogenicity-related Signal Transduction

CnPLC1 encodes a functional protein with a potential role in signalling, since hydrolysis of both PIP_2 and, to a lesser extent of PI, is reduced in Δ*plc1* (13). Similarly, recombinant CnPlc1 preferentially hydrolyses PIP_2 in a Ca^{2+}-dependent manner (unpublished data). Preferential hydrolysis of PIP_2 over PI would generate two intracellular secondary messengers, 1,2-diacylglycerol (DAG) (the sole hydrolysis product of PI) and inositol 1,4,5-trisphosphate (IP_3). Preference for hydrolysing PIP_2 is often indicative of a lack of preference for hydrolysis of GPI and suggests that the reduction in Plb1 secretion in Δ*plc1* is most likely controlled by Plc1-dependent signalling mechanisms. Finally, DAG generated by the sphingolipid biosynthetic pathway activates the sole protein kinase C isoform (Pkc1), which, in *C. neoformans* and *S. cerevisiae*, is an integral component of the mitogen-activated protein kinase (Mpk1) pathway (15) and allows laccase sequestration and melanisation within the cell wall (16).

Plc1 and Signalling Through the Calcineurin and Pkc/Mpk1 Pathways

In contrast to *S. cerevisiae*, the calcineurin and Pkc/Mpk1 signalling pathways coordinately regulate high temperature growth and cell wall integrity in *C. neoformans* (15). As Plc1 is essential for both phenotypes and can hydrolyse PIP2 to produce established activators of both pathways, Plc1 may represent the potential link to both pathways (**Fig. 1**). In agreement with Plc1 requirement for signalling through the Pkc/Mpk1 pathway, the terminal kinase Mpk1 in Δ*plc1* was not activated by phosphorylation following cell wall perturbation (13). Plc1-dependent activation of Mpk1 may or may not be via DAG activation of Pkc1 (**Fig. 1**). Plc1 could potentially be linked to the calcineurin pathway via the production of IP_3, which in turn raises the levels of intracellular Ca^{2+}, causing activation of calmodulin (Cam1). Ca^{2+}-bound Cam1 subsequently activates the serine/threonine phosphatase calcineurin (15). Calcineurin is inhibited when the immunosuppressive drugs, cyclosporine A and FK506, which are also antifungal, bind to cyclophilin A and FK506 binding protein (FKBP12), respectively (15). Comparison of the transcriptome and proteome of wildtype and Δplc1 revealed a link between Plc1 and the calcineurin pathway. As in a calcineurin mutant (15), mRNA levels of FKS1, which encodes a β-1,3-glucan synthase component essential for cell wall synthesis, were increased, and FKBP12 and Cnb1 were differentially abundant, in Δplc1 (unpublished data).

Although the Pkc/Mpk1 and calcineurin signaling pathways coordinate high temperature growth and cell

wall integrity, the relative contribution of Plc1-mediated PIP_2 hydrolysis to the activation of each pathway remains to be determined using epistasis studies and inhibitors of Plc1 and calcineurin. As calcineurin has also been implicated in vesicle fusion and cytoskeletal organisation during yeast hyphal elongation, further phenotypic analysis of calcineurin deletion mutants is warranted to determine whether secretion of Plb1 is mediated via Plc1 linkage to the calcineurin pathway.

Plc1 and Signalling through cAMP/PKA Pathway

In *S. cerevisiae*, there is evidence that Plc1 signals through cAMP-regulated pathways. Firstly, Plc1 modulates interaction of the putative nutrient sensing G-protein coupled receptor, Gpr1p, with the Gα protein, Gpa2p, effecting cAMP-regulated filamentation (17). Secondly, cAMP synthesis is decreased in Δ*plc1* (18). In *C. neoformans*, a protein complex of Gpr4p, Gpa1p and Crg2p, functions to control cAMP signaling via PKA (15). This cAMP/PKA

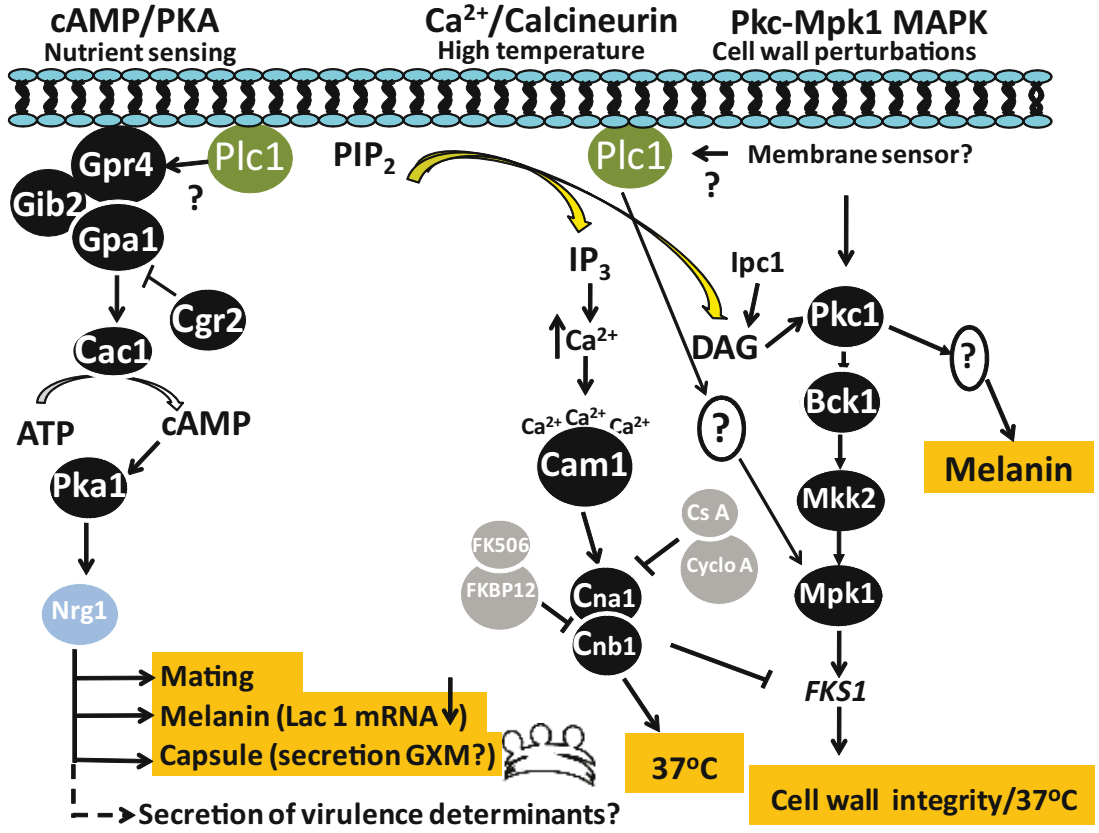

Fig. 1. Proposed role of Plc1 in pathogenicity-related signalling in *Cryptococcus neoformans*.

1. *Cyclic AMP (cAMP)/Protein kinase A (PKA) signaling pathway.* Adenylyl cyclase (Cac1) is activated by the Gα subunit (Gpa1) 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. Gpr4, G-protein-coupled receptor; Gib2, Gβ subunit. Based on studies in *S. cerevisiae* and our own published and preliminary data in *C. neoformans*, including the necessity for Plc1 in melanin production, mating and secretion of virulence determinants (13), Plc1 is linked to cAMP signaling possibly by regulating the activation of Gpa1. Lac1: laccase 1; GXM: glucuronic acid-xylose-mannan.
2. *Ca^{2+}/calcineurin signalling pathway.* This pathway primarily regulates high temperature growth in *C. neoformans*, but not in *S. cerevisiae*. High temperatures may trigger Plc1 to hydrolyse PIP_2, producing IP_3, which raises intracellular Ca^{2+} by regulating Ca^{2+} pumps/channels in the plasma membrane and ER. Cytosolic Ca^{2+} 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. Calmodulin may also act independently of the calcineurin complex. Cyclosporine A (CsA) and FK506, bind cyclophilin A and FKBP12, respectively, to inhibit calcineurin; PIP_2, phosphatidylinositol 4,5-bis phosphate. Calcineurin is also essential for hyphal elongation during mating and monokaryotic fruiting (not shown).
3. *Pkc/Mpk1 MAPK signaling pathway.* Plc1 is essential for activation of Mpk1 in response to cell wall perturbations sensed by unknown membrane proteins (13). Mpk1 activation may or may not be mediated by activation of Pkc1 via the Plc1 hydrolysis product, DAG. Pkc1 phosphorylates MAPKKK (Bck1) and subsequent phosphorylations of MAPKK (Mkk2) by Bck1 and MAPK (Mpk1) by Mkk2 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 Plc1, also activates Pkc1 to regulate melanin production independently of Mpk1 activation (20). Figure is modified from (15).

pathway regulates melanin and capsule production, and mating in response to changes in extracellular nutrients (**Fig. 1**). Δ*plc1* has a melanin defect (13), an inability to mate and reduces production of cAMP in response to glucose (unpublished data), suggesting Plc1 is linked to this signaling pathway. Comparative transcriptomic analysis of *C. neoformans* PKA mutants and wildtype identifies a secretion defect (19), further supporting the hypothesis that Plc1 is linked to the cAMP/PKA pathway, as Plb1 secretion was defective in Δ*plc1* (13). A comparison of secreted Plb1 in PKA mutants and wildtype will confirm whether this pathway does in fact play a role in secretion of pathogenicity determinants.

Plc1 as an Antifungal Drug Target

In light of the paucity of antifungal drug classes, the increasing incidence of infection relapse and drug resistance, and unfavourable toxicity/bioavailability profiles of currently marketed antifungals, investigation of alternative classes of antifungal drugs as stand-alone or synergistic therapies is urgently needed. Fungal Plc1 enzymes represent an excellent antifungal drug target as, even in the highly-adaptive *C. neoformans*, it is essential for so many pathogenic traits and adaptation to host temperature growth. Additionally, fungal Plcs differ from the mammalian Plc-δ isoform suggesting that their mode of regulation is unique. The elucidation of Plc1 function within the model fungal pathogen, *C. neoformans*, fills some essential gaps within our understanding of signalling networks of pathogenic fungi, which can differ substantially from those of *S. cerevisiae*, permits investigation into whether three individual signaling pathways constitute a regulatory network in which Plc1 plays a central role, and invites investigation of similar pathways in other pathogenic fungi. Our findings are also of importance to researchers outside the field of fungal pathogenesis as, in addition to pathogenic fungi, PLC enzymes are pathogenic determinants of bacteria and parasites, including malaria.

References

1. Park, B.J., Wannemuehler, K.A., Marston, B.J., Govender, N., Pappas, P.G., and Chiller, T.M. (2009) *AIDS* **23**, 525-530
2. Chen, J., Varma, A., Diaz, M.R., Litvintseva, A.P., Wollenberg, K.K., and Kwon-Chung, K.J. (2008) *Emerg. Infect. Dis.* **14**, 755-762
3. Theiss, S., Ishdorj, G., Brenot, A., Kretschmar, M., Lan, C.Y., Nichterlein, T., Hacker, J., Nigam, S., Agabian, N., and Kohler, G.A. (2006) *Int. J. Med. Microbiol.* **296**, 405-420
4. Falkow, S. (1988) *Rev. Infect. Dis.* **10 Suppl 2**, S274-276
5. Djordjevic, J.T., Del Poeta, M., Sorrell, T.C., Turner, K.M., and Wright, L.C. (2005) *Biochem. J.* **389**, 803-812
6. Ghannoum, M.A. (2000) *Clin. Microbiol. Rev.* **13**, 122-143
7. Cox, G.M., McDade, H.C., Chen, S.C., Tucker, S.C., Gottfredsson, M., Wright, L.C., Sorrell, T.C., Leidich, S.D., Casadevall, A., Ghannoum, M.A., and Perfect, J.R. (2001) *Mol. Microbiol.* **39**, 166-175
8. Noverr, M.C., Cox, G.M., Perfect, J.R., and Huffnagle, G.B. (2003) *Infect. Immun.* **71**, 1538-1547
9. Siafakas, A.R., Sorrell, T.C., Wright, L.C., Wilson, C., Larsen, M., Boadle, R., Williamson, P.R., and Djordjevic, J.T. (2007) *J. Biol. Chem.* **282**, 37508-37514
10. Santangelo, R.T., Chen, S.C., Sorrell, T.C., and Wright, L.C. (2005) *Med. Mycol.* **43**, 335-341
11. Feldmesser, M., Kress, Y., Novikoff, P., and Casadevall, A. (2000) *Infect. Immun.* **68**, 4225-4237
12. Rodrigues, M.L., Nakayasu, E.S., Oliveira, D.L., Nimrichter, L., Nosanchuk, J.D., Almeida, I.C., and Casadevall, A. (2008) *Eukaryot. Cell* **7**, 58-67
13. Chayakulkeeree, M., Sorrell, T.C., Siafakas, A.R., Wilson, C.F., Pantarat, N., Gerik, K.J., Boadle, R., and Djordjevic, J.T. (2008) *Mol. Microbiol.* **69**, 809-826
14. Kunze, D., Melzer, I., Bennett, D., Sanglard, D., MacCallum, D., Norskau, J., Coleman, D.C., Odds, F.C., Schafer, W., and Hube, B. (2005) *Microbiol.* **151**, 3381-3394
15. Kozubowski, L., Lee, S.C., and Heitman, J. (2009) *Cell. Microbiol.* **11**, 370-380
16. Heung, L.J., Luberto, C., Plowden, A., Hannun, Y.A., and Del Poeta, M. (2004) *J. Biol. Chem.* **279**, 21144-21153
17. Ansari, K., Martin, S., Farkasovsky, M., Ehbrecht, I.M., and Kuntzel, H. (1999) *J. Biol. Chem.* **274**, 30052-30058
18. Demczuk, A., Guha, N., Nguyen, P.H., Desai, P., Chang, J., Guzinska, K., Rollins, J., Ghosh, C.C., Goodwin, L., and Vancura, A. (2008) *Eukaryot. Cell* **7**, 967-979
19. Hu, G., Steen, B.R., Lian, T., Sham, A.P., Tam, N., Tangen, K.L., and Kronstad, J.W. (2007) *PLoS Pathog.* **3**, e42
20. Heung, L.J., Luberto, C., Plowden, A., Hannun, Y.A., and Del Poeta, M. (2004) *J. Biol. Chem.* **279**, 21144-21153