Fungal Inositol Pyrophosphate IP$_7$ Is Crucial for Metabolic Adaptation to the Host Environment and Pathogenicity

Sophie Lev, Cecilia Li, Desmarini Desmarini, Adolfo Saiardi, Nicole L. Fewings, Stephen D. Schibeci, Raghwa Sharma, Tanja C. Sorrell, Julianne T. Djordjevic

Centre for Infectious Diseases and Microbiology, Westmead Millennium Institute, Westmead, and Sydney Medical School-Westmead, University of Sydney, New South Wales, Australia; Medical Research Council Laboratory for Molecular Cell Biology, University College London, London, United Kingdom; Centre for Immunology and Allergy Research, Westmead Millennium Institute, Westmead, New South Wales, Australia; Anatomical Pathology, Westmead Hospital, Westmead, and Universities of Sydney and Western Sydney, New South Wales, Australia; Marie Bashir Institute for Infectious Diseases and Biosecurity, University of Sydney, New South Wales, Australia

ABSTRACT Inositol pyrophosphates (PP-IPs) comprising inositol, phosphate, and pyrophosphate (PP) are essential for multiple functions in eukaryotes. Their role in fungal pathogens has never been addressed. Cryptococcus neoformans is a model pathogenic fungus causing life-threatening meningoencephalitis. We investigate the cryptococcal kinases responsible for the production of PP-IPs (IP$_7$/IP$_8$) and the hierarchy of PP-IP importance in pathogenicity. Using gene deletion analysis and inositol pyrophosphate profiling, we identified Kcs1 as the major IP$_6$ kinase (producing IP$_7$) and Asp1 as an IP$_7$ kinase (producing IP$_8$). We show that Kcs1-derived IP$_7$, but not Asp1-derived IP$_8$, is crucial for pathogenicity. Global expression profiling and carbon source utilization testing suggest that IP$_7$-deficient cryptococci cannot adapt their metabolism to allow growth in the glucose-poor environment of the host lung is due to its inability to utilize alternative carbon sources. Despite this metabolic defect, the Δkcs1 mutant established persistent, low-level asymptomatic pulmonary infection but failed to elicit a strong immune response in vivo and in vitro and was not readily phagocytosed by primary or immortalized monocytes. Reduced recognition of the Δkcs1 cells by monocytes correlated with reduced exposure of mannoproteins on the Δkcs1 mutant cell surface. We conclude that IP$_7$ is essential for fungal metabolic adaptation to the host environment, immune recognition, and pathogenicity.

IMPORTANCE Cryptococcus neoformans is responsible for 1 million cases of AIDS-associated meningitis and ~600,000 deaths annually. Understanding cellular pathways responsible for pathogenicity might have an impact on new drug development. We characterized the inositol pyrophosphate kinases Kcs1 and Asp1, which are predicted to catalyze the production of inositol pyrophosphates containing one or two diphosphate moieties (PP-IPs). Using gene deletion analysis and inositol pyrophosphate profiling, we confirmed that Kcs1 and Asp1 are major IP$_6$ and IP$_7$ kinases, respectively. Kcs1-derived IP$_7$, but not Asp1-derived IP$_8$, is crucial for pathogenicity. Global expression profiling and carbon source utilization testing suggest that IP$_7$-deficient cryptococci cannot adapt their metabolism to allow growth in the glucose-poor environment of the host lung, and consequently, fungal burdens are significantly reduced. Persistent asymptomatic Δkcs1 mutant infection correlated with decreased mannoprotein exposure on the Δkcs1 mutant surface and reduced phagocytosis. We conclude that IP$_7$ is crucial for the metabolic adaptation of C. neoformans to the host environment and for pathogenicity.

Cryptococcus neoformans var. grubii is a basidiomycetous opportunistic human fungal pathogen of global significance that commonly infects immunocompromised hosts, including those with AIDS. Cryptococcus neoformans is responsible for ~1 million cases of HIV-associated cryptococcal meningitis and ~600,000 deaths per year worldwide, predominantly in sub-Saharan Africa (1). Cryptococcal lung infection within an immunocompromised host can result from the inhalation of infectious propagules or the reactivation of a latent infection (2). Dissemination from the lungs to the central nervous system (CNS) occurs via the blood or lymphatic system (2). Survival of Cryptococcus neoformans within the adverse environment of the host depends on the coordinated functions of multiple stress-related signaling pathways that regulate the expression of a suite of virulence traits and remodeling of the fungal cell wall (3).

Cryptococcal virulence determinants include the ability to grow at host physiological temperature (37°C) and the production of melanin and polysaccharide capsule (for a review, see references 4 to 7). Melanin protects cells from damage caused by reactive oxygen species, which macrophages produce as part of their antimicrobial defense (8). It is deposited in the cell wall via the catalytic activity of the cell wall-associated enzyme laccase (9).
capsule surrounds the cell wall and is comprised of glucuronoxylomannan (GXM) (90%), galactoxylomannan (GalXM), and highly mannosylated and immunogenic mannoproteins (1%) (10, 11). It protects cryptococci within the host by having a suppressive effect on the host proinflammatory response, by inhibiting the antigen-presenting capability of monocytes, and by rendering cryptococcal cells resistant to phagocytosis by shielding cell wall glucans and mannoproteins from recognition by macrophages and dendritic cells (7, 12, 13). Many mannoproteins are tethered to the cell wall by a glycosylphosphatidylinositol (GPI) anchor (14) and are localized predominantly in the innermost layer of the capsule. However, they can become detached from the anchor and secreted through the capsular layer (15–19). Several of these mannoproteins are potential vaccine candidates against cryptococcal infection (14, 20–22).

The cryptococcal enzyme phospholipase C1 (Plc1) is a component of a stress-related signaling pathway governing the production of a suite of virulence traits, which are compromised when the Plc1-encoding gene is deleted (23). We demonstrated that recombinant Plc1 hydrolyzes the membrane lipid, phosphatidylinositol-bisphosphate (PIP2) to produce inositol 1,4,5-trisphosphate (IP3) (23) and that IP3 is a substrate for cryptococcal Arg1, an inositol polyphosphate kinase (IPK) (24). As with the δPlc1 mutant, multiple cryptococcal virulence traits were compromised in the Δarg1 mutant (24), suggesting that the absence of more complex inositol polyphosphates (IPs) and/or inositol pyrophosphates (PP-IPs) downstream of Arg1 contributes to the reduced virulence observed for both mutants.

PP-IPs, of which IP2 and IP4 are the most physiologically relevant, differ from IPs in that they possess a diphosphate on one or more carbons of the inositol ring. They regulate diverse cellular functions in eukaryotes (25–32). In the nonpathogenic yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, different IP2 isoforms are essential for growth, vacuole biogenesis, salt tolerance, endocytic trafficking, the function of the cortical actin cytoskeleton, surface adhesion, and dimorphic switching in S. pombe (26, 33–37). PP-IPs affect cellular function at both the transcriptional and the posttranslational level via mechanisms that remain to be fully understood. These molecules modulate protein function directly through binding or indirectly by phosphorylating prephosphorylated proteins via a nonenzymatic reaction (38, 39). IP2 isoforms can also compete with membrane PI(3,4,5)P3 for binding to pleckstrin homology domain-containing proteins, affecting the association of these proteins with the plasma membrane, as observed in Dictyostelium discoideum (25). Recently, Worley et al. (40) showed that PP-IPs act in parallel with the TORC1 pathway to control the activity of the class I histone deacetylase Rpd3L and elicit global transcriptional changes in response to different types of stress (40). PP-IPs are therefore central regulators of eukaryotic cellular function, and targeting their synthesis holds promise for the development of new antifungal therapies. However, the kinases directly responsible for the synthesis of PP-IPs in fungal pathogens have not been determined, and the direct role of PP-IPs in regulating fungal pathogenicity remains unclear.

In the present study, we identified two PP-IP-producing kinases, Kcs1 and Asp1 (homolog of S. cerevisiae Vip1) in C. neoformans, characterized their enzymatic activities, and investigated the role of their products in pathogenicity and cellular function. Since IP7 was found to be the most crucial PP-IP for cryptococcal pathogenicity, we further investigated its role in cellular function using gene expression and carbon source utilization studies.

RESULTS

Kcs1 is an IP6 kinase in C. neoformans. We identified a putative IP6 kinase-encoding gene (CNAG_02897/KCS1) in C. neoformans var. grubii (strain H99) using Kcs1 from S. cerevisiae in a homolog search. The protein encoded by KCS1 is predicted to be 1,249 amino acids long and to contain an IPK domain at the C terminus. C. neoformans Kcs1 (CnKcs1) has 53% similarity to S. cerevisiae Kcs1 (ScKcs1) at the amino acid level. While the long N-terminal sequences are highly varied among fungal Kcs1 homologs, the C-terminal IPK domains are more conserved: the ScKcs1 and CnKcs1 IPK domains share 50% similarity. Phylogenetic analysis of the fungal Kcs1 homologs using full Kcs1 sequences or IPK domains demonstrate that CnKcs1 is clustered with other Kcs1 homologs of Basidiomycetes (Fig. 1A).

Next, we assessed the role of Kcs1 in cryptococcal IP metabolism by creating a KCS1 deletion strain (Δkcs1 mutant) and comparing its IP profile with that of the wild type (WT) and Δarg1 mutant (see Text S1 and Fig. S1A and B in the supplemental material; Fig. 1B). Intracellular IPs in the WT and mutant strains were radiolabeled by adding [3H]myo-inositol to the culture medium, extracted from the cells and fractionated using anion-exchange high-performance liquid chromatography (HPLC). As in S. cerevisiae and mammalian cells, the most abundant IP species in the WT was IP6. Accumulation of IP3 in the C. neoformans Δarg1 mutant was consistent with the findings of our previous study, where raised levels of IP3 were detected using a radiometric IP3 binding assay (24). The Δarg1 mutant was also deficient in more highly phosphorylated IP species, including the predicted products of Arg1 (IP4 and IP5) and IP6 to IP8 (IP6–8). In the C. neoformans Δkcs1 mutant, the amount of IP6–8 was similar to that in the WT. However, the more highly phosphorylated PP-IP5 (IP7) and PP2-IP4 (IP8) were not detected, consistent with Kcs1 functioning as the major IP6 kinase in C. neoformans. These results also establish that Arg1 and Kcs1 are components of the same metabolic pathway.

The mammalian IP6 kinase IP6K1 has been reported to perform physiological functions that are independent of its IP6 kinase activity (41). Furthermore, CnKcs1 and ScKcs1 contain an extended N terminus, which may have additional physiological functions. To distinguish Kcs1 kinase-dependent and -independent effects, we created the Δkcs1::KCS1AAA strain, in which the sequence encoding conserved amino acids in the Kcs1 catalytic domain (1032--1034) was altered to encode AAA, resulting in the expression of kinase-inactive Kcs1 (Text S1; Fig. S1A). This same substitution abrogated Kcs1 catalytic activity in S. cerevisiae (34). The Δkcs1::KCS1AAA strain was created by ectopic insertion of the mutagenized KCS1 gene with its native promoter and terminator into the genome of the Δkcs1 mutant. A control strain (the Δkcs1::KCS1 strain) was similarly created by inserting the native KCS1 genomic fragment ectopically into the genome of the Δkcs1 mutant. Integration of KCS1AAA and KCS1 into a single genomic location in each strain was confirmed by Southern blotting (Fig. S1C). The levels of expression of ectopically integrated KCS1 and KCS1AAA in the respective reconstituted strains were comparable to the expression of native KCS1 in the WT (Fig. S1D). Like the Δkcs1 mutant, the Δkcs1::KCS1AAA strain did not produce IP7–8 (Fig. 1B, inset). The IP profile of the Δkcs1::KCS1 strain was
similar to that of the WT (Fig. 1B, inset). The Δkcs1 and Δkcs1::KCS1AAA strains were subsequently used to identify phenotypes that were specifically associated with the absence of Kcs1 catalytic activity.

Loss of IP6 kinase activity leads to growth and cell wall integrity defects. The ability of C. neoformans to colonize host tissue is dependent on its ability to grow at mammalian physiological temperatures. Since PP-IP products generated by ScKcs1 play a role in the response to heat stress, the growth of the WT, Δkcs1, and Δarg1 strains was assessed at 30°C, 37°C, and 39°C using a drop dilution test. Like that of the ScΔkcs1 mutant, the growth of the Δkcs1 and Δkcs1::KCS1AAA strains at all three temperatures was reduced compared to that of the WT and Δkcs1::KCS1 strain (Fig. 2).

We also investigated the integrity of the cell wall in the Δkcs1 and Δkcs1::KCS1AAA mutants and their ability to produce mating filaments. Cell wall integrity was assessed by comparing the extent of growth in the presence of cell wall-perturbing agents. Figure 2 demonstrates that the growth of the Δkcs1 and Δkcs1::KCS1AAA strains was similarly reduced in the presence of SDS and caffeine, compared with that of the WT and Δkcs1::KCS1 strains, consistent with a cell wall integrity defect. The Δkcs1 and Δkcs1::KCS1AAA (mating type α) strains also produced fewer mating filaments fol-

FIG 1 Kcs1 is an IP6 kinase in C. neoformans. (A) Phylogenetic analysis of Kcs1 homologs in fungi. Full-length C. neoformans Kcs1 protein (indicated by the arrow) was used to generate the phylogenetic tree. Cg, Candida glabrata XP_445605; Sc, Saccharomyces cerevisiae Q12494; Af, Aspergillus fumigatus XP_753330; An, Aspergillus nidulans XP_664486; Ci, Coccidioides immitis EAS27460.2; Um, Ustilago maydis XP_757030.1; Cc, Coprinopsis cinerea XP_001836888; Pc, Phanerochaete carnosa EKM59468; Ta, Trichosporon asahii EKD02283; Cn, Cryptococcus neoformans AFR93794. Numbers represent bootstrap values. (B) Analysis of the inositol polyphosphate profile of the WT, Δkcs1, and Δarg1 strains. The inset represents IP profiles of the reconstituted Δkcs1 strains: the Δkcs1::KCS1 strain (expressing native Kcs1) and the Δkcs1::KCS1AAA strain (expressing kinase-inactive Kcs1). Intracellular IPs were labeled with [3H]myo-inositol, extracted, and analyzed by anion-exchange HPLC. The elution time for different IP species was determined using standards. The position of each IP species is indicated.

FIG 2 Spot dilution assays demonstrating a cell wall defect in the Δkcs1 and kinase-inactive Δkcs1::KCS1AAA strains but not in the kinase-active Δkcs1::KCS1 strain. The strains were serially diluted 10-fold (106 to 10 cells/spot from left to right) and spotted onto the test plates indicated. The dual nourseothricin (Nat) and neomycin (Neo) resistance of the Δkcs1::KCS1 and Δkcs1::KCS1AAA strains confirms that integration of the KCS1 construct (providing Nat resistance) occurred in the Δkcs1 mutant strain background (Neo resistance).
following a unilateral cross with KN99 (mating type a), while filament production was restored to WT levels in the ∆kcs1::KCS1 strain (Fig. S2). Since the cell wall integrity and mating defects persisted in the ∆kcs1::KCS1 strain (Fig. 2; Fig. S2), it can be concluded that the PP-IPs produced by Kcs1 are essential for cell wall integrity and mating.

The ∆kcs1 mutant is hypersusceptible to antifungal agents. To investigate whether disruption of PP-IP synthesis alters the susceptibility of C. neoformans to antifungal drugs, the extent of growth of the WT and the ∆kcs1 mutant was compared in the presence of amphotericin B, voriconazole, itraconazole, and range of azoles (Table S1). The ∆kcs1 mutant was 8 times more susceptible than the WT to posaconazole, voriconazole, itraconazole, and fluconazole and twice as susceptible to flucytosine and amphotericin B but remained resistant to the echinocandins, caspofungin, micafungin, and anidulafungin. The ∆arg1 mutant demonstrated a similar drug susceptibility profile (Table S1 and Text S1).

The ∆kcs1 mutant has defective melanin production and an increased capsule size. Next, we assessed the ability of the Kcs1-deficient strains to produce melanin. Melanin protects cryptococci from a variety of stresses, including macrophage-derived oxidative stress and UV light. The ∆kcs1 and ∆kcs1::KCS1 strains produced less melanin than the WT when grown on minimal medium (MM) without glucose and supplemented with 1,3,4-dihydroxyphenylalanine (L-DOPA) (Fig. 3A). The extent of melanization in the ∆kcs1::KCS1 strain was similar to that of the WT. Since the cell wall-associated enzyme laccase 1 catalyzes melanin synthesis in C. neoformans, allowing it to be deposited in the cell wall, the extracellular laccase activity associated with intact fungal cells was also measured. The results in Fig. 3B demonstrate that the reduced melanization of the ∆kcs1 and ∆kcs1::KCS1 strains (Fig. 3A) coincided with reduced extracellular laccase activity as measured by oxidation of the laccase substrate 2,2’-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS). Once again, laccase activity was restored to WT levels in the ∆kcs1::KCS1 strain. To determine whether reduced laccase activity in the Kcs1-deficient strains is due to the absence of LAC1 gene induction under melanin-inducing conditions, we measured LAC1 expression in the WT, ∆kcs1, ∆kcs1::KCS1, and ∆kcs1::KCS1 strains following 5 h of growth in MM without glucose. Figure 3C demonstrates that the LAC1 expression profile in all strains is similar to that observed for laccase activity and melanin production. These data indicate that Kcs1-derived PP-IPs are essential for induction of LAC1.

Capsule production was also measured following the growth of fungal strains on Sabouraud (SAB) agar. Notably, colonies formed by the Kcs1-deficient strains had a mucoid (glossy) appearance, in contrast to the dull surface of the WT (Fig. 4A). The capsules produced by the ∆kcs1 and ∆kcs1::KCS1 strains were on average larger than those produced by the WT (Fig. 4B and C). Colony appearance and capsule size were partially restored in the ∆kcs1::KCS1 strain.

Phenotypic defects in the ∆kcs1 mutant are caused by the absence of IP7, not IP8. Kcs1 is essential for the production of the PP-IPs IP7 and IP8. To determine the relative contribution of IP8 to the Kcs1-dependent traits, we identified a homolog of the IP6–7 kinase of S. cerevisiae (ScVip1) in C. neoformans, designated Asp1. Asp1 protein (encoded by CNAG_02161)
is predicted to be 1,117 amino acids long and is 33% identical and 24% similar to ScVip1. Like ScVip1 and Asp1 in *Schizosaccharomyces pombe*, Asp1 of *C. neoformans* contains an N-terminal ATP grasp domain responsible for the kinase activity and a C-terminal histidine acid phosphatase domain of unknown function. *C. neoformans* ASP1 was deleted as described for *KCS1*, creating the \( \Delta asp1 \) mutant. IP\(_7\) accumulated and PP\(_2\)-IP\(_4\) (IP\(_8\)) was not detected in the \( \Delta asp1 \) mutant (Fig. 5), consistent with *C. neoformans* Asp1 functioning as an IP\(_3\) kinase, as reported for *S. cerevisiae* Vip1 (42, 43). A reduction in IP\(_3\) and IP\(_5\) was also noted in the \( \Delta asp1 \) strain (Fig. 5). The growth of the \( \Delta asp1 \) mutant was assessed under the same conditions as those for the \( \Delta kcs1 \) mutant, including 37°C, in the presence of cell wall-perturbing agents, and on mating agar (Fig. S3). The \( \Delta asp1 \) strain was also assessed for the production of melanin and capsule. Under all growth conditions, the \( \Delta asp1 \) strain exhibited a phenotype similar to that of the WT, indicating that Kcs1-derived PP-IP\(_5\) (IP\(_7\)), rather than Asp1-derived PP\(_2\)-IP\(_4\) (IP\(_8\)), is the most crucial PP-IP for cryptococcal cellular function.

The \( \Delta kcs1 \) mutant is less pathogenic than the WT in animal models. The pathogenicity of the \( \Delta kcs1 \) mutant was compared to that of the WT in an invertebrate model, the greater wax moth (*Galleria mellonella*), and a mouse inhalation model. Larvae of *Galleria mellonella* were infected by inoculation of fungi into the lower proleg and incubated at 30°C. The average survival of \( \Delta kcs1 \) mutant-infected larvae was 14 days, compared with 5 days for WT-infected larvae (Fig. 6A). Mice were inoculated via the nares. The median survival time of mice infected with the WT was 14 days. However, no mice succumbed to infection with the \( \Delta asp1 \) mutant for up to 50 days postinoculation (Fig. 6B). Virulence was almost restored by the introduction of *KCS1* into the \( \Delta kcs1 \) mutant, with the median survival time of \( \Delta kcs1::KCS1 \) strain-infected mice being 18 days. Despite the avirulent phenotype, \( \Delta kcs1 \) cells were detected in mouse lungs (3 × 10\(^5\) to 90 × 10\(^5\) CFU per total lung tissue) over the 50-day postinfection period (Fig. 6C). The \( \Delta kcs1 \) mutant infection burden was ~8-fold lower than that of the WT at 3 days postinfection. However, the difference between the WT and the \( \Delta kcs1 \) mutant pulmonary burdens increased to ~140-fold at the onset of the inflammatory response 7 days postinfection (see Fig. 7) and to ~286-fold 14 days postinfection. These increases were due primarily to the more rapid increase in WT pulmonary burden than in \( \Delta kcs1 \) mutant burden over this time. Despite the persistence of \( \Delta kcs1 \) mutant in lungs up to 50 days postinfection, no colonization of the brain was observed during this time (Fig. 6D). This was in contrast to what occurred with mice infected with the WT, which developed a CNS infection by day 14 (the median survival time of WT-infected mice).

Periodic acid-Schiff (PAS)-stained sections of WT- and \( \Delta kcs1 \) mutant-infected lungs at day 7 and day 14 are shown in Fig. S4 in the online edition.
A dense WT infection was observed on both days within the alveolar spaces and bronchi, with evidence of extensive infiltration of monocytes and polymorphs. In contrast, but consistent with organ burden analysis, \( \Delta kcs1 \) mutant infection was sparse and lung tissue was healthier than in WT-infected lung, as indicated by large areas of well-aerated alveoli and minimal evidence of inflammation. Despite the death of \( \Delta kcs1 \) mutant cells over the infection time course, budding cells were observed in the infected lung tissue.

The \( \Delta asp1 \) mutant was also tested for virulence in a mouse inhalation model, and the progression of disease in \( \Delta asp1 \) mutant-infected animals was found to be similar to that of WT-infected animals. This was expected, as no phenotypic differences were observed between the WT and the \( \Delta asp1 \) strain (Fig. S3 and S5).

\( \Delta kcs1 \) mutant infection elicits a weak host immune response. The persistent, asymptomatic \( \Delta kcs1 \) mutant infection observed in the mouse model (Fig. 6C) prompted us to compare the immune responses elicited by the WT and the \( \Delta kcs1 \) mutant. The cytokine profile in infected lung tissue at 3, 7, and 14 days postinoculation was determined by analyzing the supernatants of homogenized lung extracts prepared from WT-, \( \Delta kcs1 \) mutant-, and mock-infected animals using a cytokine bead array (CBA) and flow cytometry (Fig. 7).

WT infection elicited a strong proinflammatory response in the lung (gamma interferon [IFN-\( \gamma \)] and tumor necrosis factor [TNF]), which peaked at day 7 postinfection; the day 7 response for IFN-\( \gamma \) and TNF-\( \alpha \) was 36- and 3-fold higher than the day 3 response, respectively, and 688- and 27-fold higher than the response observed in mock-infected animals (Fig. 7). TNF is required for neutrophil recruitment into pulmonary tissue during a cryptococcal infection (44), and IFN-\( \gamma \) maintains monocyte-derived macrophages in an activated state conducive to fungal

FIG 6 Ksc1 is essential for pathogenicity in \textit{Galleria mellonella} and mice. (A) \textit{G. mellonella} larvae were inoculated with the WT or the \( \Delta kcs1 \) mutant (10^6 yeast cells/10\( \mu \)l). Two groups of larvae were also mock infected with water, or not at all, to serve as controls. The larvae were incubated at 30°C for 10 days, their health was monitored, and their deaths were recorded. (B to D) Anesthetized mice were inoculated intranasally with 5 \( \times \) 10^5 CFU/20 \( \mu \)l of the indicated strains and euthanized after exhibiting debilitating symptoms of infection for the survival study (B) or at 3, 7, and 14 days postinfection for organ burden analysis (C and D). (A and B) \( \Delta kcs1 \) mutant-infected animals not showing clinical symptoms of illness were culled at days 15 and 50, and censorship of these infection groups is indicated by the cross. The Kaplan-Meier log rank test was used to determine differences in survival, with \( \Delta kcs1 \) mutant-infected larvae and mice surviving longer than WT-infected animals and the reconstituted strain in the case of the mouse study (\( *, P < 0.05 \)). The difference in survival of mice infected with the WT and \( \Delta kcs1::KCS1 \) strain was insignificant (\( P = 0.161 \)). Lungs (C) and brain (D) were removed and homogenized for organ burden analysis (CFU per organ) using quantitative culture.
killing and clearance. The strong proinflammatory response in WT-infected mice was accompanied by a strong monocyte chemotactic protein-1 (MCP-1) response, which also peaked at day 7. MCP-1 is a crucial mediator of monocyte and T cell recruitment to the sites of infection. At day 7, the MCP-1 response was 22-fold higher than the day 3 response and 40-fold higher than the response observed in mock-infected animals. Despite the decrease in MCP-1 levels at day 14, significant numbers of monocytes were present at this time point in WT-infected lung (Fig. S4). In contrast to WT, no increase in the level of any of these three cytokines was observed in Δkcs1 mutant-infected lung at 7 days, despite evidence of persistent Δkcs1 mutant infection. Interleukin 6 (IL-6) production also peaked in WT-infected lung at day 7, with the response being ~4-fold higher than at day 3 and ~84-fold higher than the response observed in mock-infected animals. In contrast to the proinflammatory cytokines and MCP-1, IL-6 production peaked at day 7 in Δkcs1 mutant-infected lungs and was ~36-fold higher than the day 3 response and ~17-fold higher than the response observed in mock-infected animals.

IL-2 levels increased in Δkcs1 mutant-infected lungs over the time course but particularly from day 3 to day 7. However, the day 3 level was lower than the mock-infected control and WT values, while the day 7 level was similar to that of the mock-infected control but lower than the WT value. Interestingly, similar IL-2 responses were obtained for WT- and Δkcs1 mutant-infected lungs at day 14 despite the significantly lower Δkcs1 mutant fungal burden. In contrast to that of the other cytokines, IL-4 and IL-17-A production was higher at day 14 than at day 7 in WT-infected lungs. However, the IL-4 response observed for Δkcs1 mutant-infected lungs did not change significantly between days 7 and 14 but was still higher than the response observed in mock-infected lungs (Fig. 7).

For both the WT and Δkcs1 strains, IL-17A levels at day 3 were below the level of detection and are not shown. At day 7, the IL-17A level in Δkcs1 mutant–infected lung was lower than that in WT-infected lung, while at day 14, a similar IL-17A response occurred in WT- and Δkcs1 mutant-infected lungs, despite the significantly lower Δkcs1 mutant burden.

The phagocytosis of the Δkcs1 mutant by monocytes and subsequent monocyte activation are compromised. Monocytes and macrophages are the first line of defense against cryptococcal infection. Since we observed an altered surface phenotype in the Kcs1-deficient mutants (Fig. 4A), which may affect cryptococcal recognition by monocytes, we compared the efficiencies of phagocytosis of the WT and Δkcs1 mutant in vitro, using the THP-1 monocytic cell line. Fungal cells were labeled with fluorescein isothiocyanate (FITC) and opsonized with human serum (containing complement and antifungal antibodies), and their uptake was measured by flow cytometry. Figure 8A demonstrates that after 4 h of incubation, ~40% of THP-1 cells were associated with WT C. neoformans. However, only ~10% of THP-1 cells were associated with Δkcs1 cells. Figure S6A shows a representative scatterplot used for quantification of adhesion/uptake. The extent of THP-1 cell association with the WT and Δkcs1 mutant was also measured when IgG antibodies purified from human blood were used as the opsonizing agent. The results in Fig. 8A show that only ~20% of THP-1 cells were associated with WT C. neoformans, indicating that the combination of complement and antibody is a more potent opsonin of WT cryptococci than antibody alone. As with the results observed for serum opsonization, the number of THP-1 cells associated with IgG-opsonized Δkcs1 cells was reduced. Notably, the difference in THP-1 cell association between the WT and mutant was even greater when IgG was used as an opsonin than when serum was used (9-fold versus 4-fold). The extent of THP-1 cell association with the WT and Δkcs1 strains was also measured when anticapsular antibodies were used as the opsonizing agent (Fig. 8A). However, in this case, the extents of THP-1 association with the WT and Δkcs1 mutant were similar.

To rule out the possibility that Δkcs1 cells are taken up less by THP-1 monocytes due to their lower rate of proliferation, we compared the phagocytosis of heat-killed WT and Δkcs1 mutant. As with live cryptococci, the phagocytosis of dead Δkcs1 cells was
reduced (Fig. 8B). The extents of adhesion/uptake of heat-killed cryptococcal cells were also compared in primary human CD14-positive monocytes in a peripheral blood mononuclear cell (PBMC) preparation. Monocytes were distinguished from T and B cells by the binding of fluorescent anti-CD14. As with THP-1 cells (Fig. 8A and B), monocyte uptake of heat-killed, serum-opsonized Δkcs1 cells by CD14-positive monocytes was reduced (Fig. 8C). Microscopy of the cocultures also revealed reduced association of the Δkcs1 mutant with THP-1 cells and PBMCs (Fig. S6B), consistent with the flow cytometry analysis of fungal adhesion/uptake.

To determine whether reduced phagocytosis of Δkcs1 cells by monocytes coincided with reduced monocyte activation, we compared levels of THP-1 activation in the presence of live WT or Δkcs1 cells after 4 h of coincubation, using an RT2 profiler PCR array. The results in Fig. 8D indicate that the expression of proinflammatory chemokine- and interleukin-encoding genes in activated THP-1 cells following coculture with serum-opsonized WT and Δkcs1 cells was quantified using an RT2 profiler PCR array designed for analysis of the antifungal response. The expression data were normalized to the expression of the housekeeping genes indicated and then calculated as the fold change from the expression of activated THP-1 cells that had not been coincubated with C. neoformans. (E) Each culture was incubated with ConA-FITC and the fluorescence quantified using flow cytometry. The bar graph represents the average fluorescence of WT and Δkcs1 cells. Except for those in panel D, all results represent the means and standard deviations of results from biological triplicates. *, P < 0.005 using an unpaired, two-tailed t test.
TABLE 1 The expression of genes encoding extracellular proteins is lower in Δkcs1 cells than in WT cells

| Identifier   | Description                                | Relative expression of Δkcs1 cells compared to WT cells (fold change) | Presence of O-linked glycosylation | Presence of N-linked glycosylation | GPI   |
|--------------|--------------------------------------------|--------------------------------------------------------------------|-----------------------------------|-----------------------------------|-------|
| CNAG_06291   | Poly saccharide deactylase Fpd1 (D25)       | 410.5                                                               | +                                 | +                                 |       |
| CNAG_03465   | Laccase                                    | 99.52                                                               | 8.91                              | +                                 |       |
| CNAG_06346   | Liprotein A                                | 180.3                                                               | 10.7                              | +                                 |       |
| CNAG_05411   | Endoglucanase                              | 33.53                                                               | 10.7                              | +                                 |       |
| CNAG_06260   | α-Glucosidase                              | 67.41                                                               | 10.7                              | +                                 |       |
| CNAG_05918   | ␤-Glucosidase                              | 42.12                                                               | 10.7                              | +                                 |       |
| CNAG_06213   | Pepsin, aspartic protease                   | 176.5                                                               | 10.7                              | +                                 |       |
| CNAG_04380   | Aspartic peptidase                         | 177.4                                                               | 10.7                              | +                                 |       |
| CNAG_00488   | Phospholipase B1-like lipase                | 180.8                                                               | 10.7                              | +                                 |       |
| CNAG_01040   | Carboxypeptidase D                         | 187.7                                                               | 10.7                              | +                                 |       |
| CNAG_07638   | PLC-like phosphodiesterase                 | 187.7                                                               | 10.7                              | +                                 |       |
| CNAG_09581   | Aspartic peptidase                         | 187.7                                                               | 10.7                              | +                                 |       |
| CNAG_00573   | Ser/Thr protein phosphatase                | 187.7                                                               | 10.7                              | +                                 |       |
| CNAG_07600   | α-Glucosidase                              | 187.7                                                               | 10.7                              | +                                 |       |
| CNAG_04625   | Serine-type endopeptidase                  | 187.7                                                               | 10.7                              | +                                 |       |
| CNAG_05471   | α-Glucosidase                              | 187.7                                                               | 10.7                              | +                                 |       |
| CNAG_05473   | Serine carboxypeptidase                    | 187.7                                                               | 10.7                              | +                                 |       |
| CNAG_07866   | Ferro-O2-oxidoreductase                    | 187.7                                                               | 10.7                              | +                                 |       |

*Normalized fpkm values (fragments per kilobase of exon per million reads mapped) were generated by the Galaxy-based Cuffdiff tool and used as a measure of gene expression.*

Flammarol cytokine- and interleukin-encoding genes is significantly higher in THP-1 cells coincubated with WT than with Δkcs1 cells, consistent with the stronger cytokine induction profile observed in the WT-infected mouse lung (Fig. 7). For example, the induction of the gene encoding the monocyte chemotactant protein (Mcp1/Ccl2) was >9-fold higher in THP-1 cells coincubated with the WT than in cells coincubated with the Δkcs1 mutant. Thus, the reduced rate of Δkcs1 cell phagocytosis correlated with reduced monocyte activation.

Mannoprotein exposure at the Δkcs1 cell surface is reduced. The similar rates of phagocytosis of anti-GXM-opsonized WT and Δkcs1 cells suggest that the antibody binds similarly to the surfaces of both strains despite their microscopic (larger capsule size) and macroscopic (mucoid colony morphology) differences. The difference in phagocytosis of WT and Δkcs1 cells observed when serum or human IgG were used for opsonization suggests that the respective opsonins bind to surface epitopes other than the sugar moiety binding targets of anti-GXM antibodies. Furthermore, the data suggest that the targets of serum and human IgG opsonins are present in greater amounts on WT than on Δkcs1 cells. The capsule contains mannoproteins, in addition to polysaccharides, and human serum contains antibodies against fungal mannoproteins that can initiate classical pathway activation upon binding to macropages (45). To investigate mannoprotein exposure at the surfaces of WT and Δkcs1 cells, the extents of concanavalin A (ConA) binding were compared. ConA is a lectin that reacts with surface mannose sugars found on many microbes and is available as an FITC conjugate so that binding can be assessed by flow cytometry. The results in Fig. 8E show that ConA-FITC binding to the Δkcs1 cell surface is decreased in comparison with binding to WT cells, suggesting that mannosylated proteins are not displayed normally on the surface of the mutant cells.

To determine whether the reduced presence of mannoproteins on the Δkcs1 cell surface correlates with reduced expression of genes encoding mannoproteins, we performed transcriptome sequencing (RNA-seq) of the WT and Δkcs1 strains. We focused on genes encoding proteins with a signal peptide which targets them to the endoplasmic reticulum (ER)/Golgi complex and then to the cell periphery, excluding transmembranal proteins and proteins likely to be mitochondrial, peroxisomal, or ER resident. The expression of 22 extracellular proteins was down-regulated in the mutant, with 17 predicted to be glycosylated and 4 containing a GPI anchor (Table 1). Taken together, the data indicate that Kcs1-derived IP7 affects the fungal surface composite at the transcriptional level, influencing the extent of opsonization and phagocytosis and the ensuing immune response.

Transcriptome analysis identifies metabolic changes in the Δkcs1 mutant. The gene expression profiles of the WT and Δkcs1 strains obtained by RNA-seq were further analyzed to identify changes in gene expression that could be responsible for the altered phenotype and virulence profile of the Δkcs1 mutant. Initial functional classification of differentially expressed genes was made using gene ontology (GO) term assignment generated by the Joint Genome Institute (JGI) and then refined further based on published literature and by performing a homology search. We found that the absence of IP7 resulted in an 11-fold reduction in the expression of LAC1 gene involved in melanization, consistent with the results in Fig. 3. However, the most significant changes were observed in metabolic function. Tables 2 and S3 demonstrate that genes encoding proteins involved in glycolysis are more highly expressed in the Δkcs1 mutant than in the WT. Genes involved in mRNA translation and ribosomal biosynthesis are also more highly expressed in the Δkcs1 mutant. However, expression of genes encoding numerous sugar transporters and
enzymes that comprise metabolic pathways involved in the utilization of alternative carbon sources (citric acid and glyoxylate cycles, gluconeogenesis, and fatty acid β-oxidation) is reduced in the Δkes1 mutant. These metabolic differences suggest that WT, but not Δkes1, cells actively express genes involved in the utilization of carbon sources other than glucose, including lactate and fatty acids, which require mitochondrial function.

The environment of the host lung is low in glucose (46), and enzymes involved in the use of alternative carbon sources are essential for cryptococcal pathogenicity (47–49). We therefore compared the extent of growth of both strains on glucose and three nonfermentable carbon sources, lactate, glycerol, and oleic acid, each present as the sole carbon source. We also used minimal medium (MM), which is more physiological than yeast extract-peptone-dextrose (YPD) (Fig. 2). In MM supplemented with glucose Δkes1 mutant grew similar to the WT strain (Fig. 9A). Glycerol is incorporated into the glycolytic/gluconeogenesis pathway following conversion to dihydroacetone phosphate by mitochondrial flavin adenine dinucleotide (FAD)-dependent glycerol-3-phosphate dehydrogenase. Lactate is converted to pyruvate, which is channelled into the citric acid cycle and gluconeogenesis. Oleic acid undergoes β-oxidation in mitochondria and/or peroxisomes to generate acetyl coenzyme A (acetyl-CoA), which can be used for energy generation via the citric acid cycle. The results in Fig. 9A demonstrate that in contrast to that on glucose, the growth of Δkes1 cells on the alternative carbon sources was severely compromised.

Using quantitative PCR (qPCR) and minimal medium containing glucose or lactate, we compared the levels of expression of a gene encoding a key enzyme in gluconeogenesis, phosphoenolpyruvate carboxykinase (PCK1; CNAG_042117) (Fig. 9B). Pck1 catalyzes an irreversible step of gluconeogenesis, and its expression was 20-fold lower in Δkes1 cells grown overnight in YPD than in WT cells under the same conditions (Table S3). In minimal medium supplemented with glucose, PCK1 expression was 4.5-fold lower in Δkes1 than in WT cells (Fig. 9B), consistent with the global expression data. In the absence of glucose (minimal medium with lactate), PCK1 was induced in both strains, but its expression was 2-fold lower in the Δkes1 mutant, indicating that gluconeogenesis is not fully activated in the absence of IP7. This result confirms that the mutant is compromised in its ability to metabolize alternative carbon sources and that the defect is caused, at least in part, by inadequate transcriptional regulation of metabolic enzymes.
Role of IP₇ in Fungal Pathogenicity

FIG 10 Schematic representation of the inositol polyphosphate biosynthesis pathway in C. neoformans. Phospholipase C1 (Plc1) hydrolyzes PIP₂ to produce IP₃, Arg1 is a dual-specificity kinase converting IP₃ to IP₄ and IP₅ to IP₇. Ipkl is the proposed IP₆ kinase, providing IP₈ as a substrate for Kcs1. Kcs1 is the major IP₆ kinase generating PP-IP₅ (IP₇), and Asp1 is an IP₇ kinase generating PP-2-IP₄ (IP₈). The physiological roles of Kcs1-derived IP₇ are indicated.

tectable amounts of IP₇ (or IP₈). We also showed that Asp1 functions primarily as an IP₇ kinase, converting Kcs1-generated IP₇ to IP₈. In S. cerevisiae, IP₇ is also produced by an Asp1 homolog, Vip1 (42). However, our IP profiling revealed that Asp1 does not contribute significantly to IP₇ production in C. neoformans. Based on the IP profile obtained for the Δarg1, Δkcs1, and Δasp1 mutants, we propose a model of the IP biosynthesis pathway in C. neoformans depicted in Fig. 10. The presence of a putative IP₇ kinase (Ipkl) in the middle of the pathway can be inferred from its homology to Ipkl in S. cerevisiae: its role in C. neoformans is currently being investigated in our laboratory.

The IP biosynthetic pathway as a drug target. Two observations support investigation of the fungal PP-IP biosynthetic pathway as a potential antifungal drug target: (i) the absence of redundancy in the fungal pathway compared with the mammalian pathway and (ii) low sequence similarity between fungal and mammalian enzymes. For example, while Arg1 in C. neoformans is the only enzyme able to convert IP₃ to IP₄, three IP₇ kinases isoforms and the inositol polyphosphate multikinase (IPMK) catalyze the reaction in humans. Arg1 differs structurally and functionally from mammalian IP₇ kinases, implying potential selectivity of inhibition (50, 51). Arg1 and IPMK are only 19% identical, while Kcs1 is even less similar (13% identity) to its mammalian equivalent, inositol hexakisphosphate kinase 1 isoform 1. The hypersusceptibility of Δarg1 and Δkcs1 cells to antifungal agents (Table S1) also suggests that inhibitors of fungal Kcs1 or Arg1 may act synergistically with these marketed antifungal drugs to provide a better treatment outcome.

Kcs1-derived IP₇, but not Asp1-derived IP₈, is required for optimal growth and the production of virulence traits. We observed delayed growth of the CnΔkcs1 mutant under standard growth conditions and a cell wall integrity defect but no high-temperature growth defect. In a cross with the KN99 MATa strain, the Δkcs1 mutant, but not the Δasp1 mutant, produced stunted mating filaments. Defective Δkcs1 cell walls may affect retention of the sexual agglutinins and adhesins, which are required for efficient cell contact prior to filamentation (52), as well as capsule attachment. A mucoid phenotype, typical of the Δkcs1 mutant, is likely to be associated with increased capsule size. Such a correlation was also observed in C. neoformans following phenotypic switching (53) and in the allergen 1 (ALL1) deletion mutant (54). During glucose deprivation, the Δkcs1 strain, but not the Δasp1 strain, fails to produce extracellular laccase and, consequently, melanin. We established that Kcs1-derived IP₇ affects melanization at the transcriptional level, as the gene encoding the major melanin-producing enzyme in C. neoformans, laccase 1 (LAC1), was not induced in Δkcs1 cells. Transcriptional profiling data supported this finding.

IP₇-dependent metabolic adaptation affects growth and pathogenicity. The inability of the Δkcs1 mutant to achieve high infection burdens in mouse lung is most likely multifactorial, due to its weakened cell walls, inability to produce melanin, and reduced growth rate. High-throughput analysis of gene expression in the WT and Δkcs1 mutant suggested that the Δkcs1 mutant has a reduced ability to utilize carbon sources other than glucose and that this metabolic dysfunction may be a potential reason for its reduced ability to colonize glucose-deficient mouse lungs (45, 55). It has been demonstrated that WT cryptococci harvested during pulmonary infection exhibit a gene expression profile consistent with a starvation response (47, 56, 57). Metabolic dysfunction due to the absence of IP₇, as suggested by our gene expression data, was supported by our observation of the severely reduced growth of the Δkcs1 mutant on alternative carbon sources, such as oleic acid, glycerol, and lactate (Fig. 9A). Utilization of these carbon sources requires functional mitochondria and, in the case of oleic acid, peroxisomes (58). A similar metabolic imbalance was reported for the Δkcs1 mutant of S. cerevisiae (59), whose cells did not grow on ethanol or glycerol as a sole carbon source.

Characterization of cryptococcal mutants defective in key metabolic enzymes established unequivocally the importance of β-oxidation and gluconeogenesis for the survival of C. neoformans in the nutrient-poor environment of the host and for pathogenicity. For example, a mutant in the peroxisomal β-oxidation pathway (Δmfe1 mutant) failed to grow on fatty acids as a carbon source and displayed hypovirulence in mice and reduced dissemination to the brain (49). Consistent with this report and with our observation that Δkcs1 mutant growth was reduced on oleic acid as the sole carbon source, MFE1 expression in Δkcs1 cells was 21-fold lower than in WT cells (Table S3). Similarly, a gluconeogenesis-defective cryptococcal mutant deficient in phosphoenolpyruvate carboxykinase Pck1 (Δpck1 mutant) failed to grow on lactate as a sole carbon source and was also markedly less virulent in mice (48). Figure 9B and Table S3 demonstrate that the expression of PCK1 was lower in Δkcs1 cells than in the WT; the difference was 20-fold when the WT and Δkcs1 cells were grown in YPD and 2-fold when the strains were grown in minimal medium with lactate as a sole carbon source. Taken together, our findings suggest that the reduced pathogenicity of the Δkcs1 mutant may be due to a failure to adjust its metabolic response to a nutrient-poor environment, and this failure is caused, at least in part, by compromised regulation of gene expression. Such metabolic adaptation is essential for successful host infection.

Persistence of Δkcs1 mutant infection correlates with reduced surface mannoprotein. Despite the reduced pathogenicity and metabolic defects observed in the Δkcs1 mutant, a low level of apparently asymptomatic infection persisted in vivo. Our results suggest that this persistence of Δkcs1 mutant infection in lung is due primarily to the reduction in the display of surface mannoproteins in the Δkcs1 strain, as evidenced by decreased ConA binding and reduced expression of genes encoding immunogenic mannoproteins, leading to reduced recognition by phagocytes. It is likely that the majority of the opsonic anticytophagocytic antibod-
ies in serum target mannoproteins (45, 60). Thus, the reduced mannoprotein content on the surfaces of Δkcs1 cells is expected to result in reduced opsonization by antibodies against mannoprotein and less efficient recognition of the fungal cells by phagocytic Fc receptors (45). Reduced phagocytosis of Δkcs1 cells indeed coincided with evidence of less phagocyte activation. The levels of phagocytosis of heat-killed WT and Δkcs1 cells were also compared to rule out the possibility that the reduction in phagocytosis of Δkcs1 cells by THP-1 monocytes was due to the lower proliferation rate of Δkcs1 cells. Again, the phagocytosis of killed Δkcs1 cells was reduced relative to that of WT cells (Fig. 8B). This reduction was even greater than that observed for live cells (~16-fold versus ~4-fold), possibly because a different concentration of serum was used for opsonization of the killed cells, as indicated in the legend of Fig. 8.

Persistent Δkcs1 strain lung infection may also be due to impaired sampling of mannoproteins by resident macrophages and dendritic cells for subsequent presentation to T cells, once again as a result of diminished mannoprotein production in Δkcs1 cells. Notably, the secreted polysaccharide deacetylase homolog Fpd1/D21 (CNAG_06291), which stimulates T-cell activation in mice and therefore contributes to the adaptive immune response to WT C. neoformans (61, 62), was down-regulated in Δkcs1 cells (Table 1). Transcription of many genes encoding proteases, including aspartic proteases (CNAG_00213, CNAG_04380, and CNAG_00581), carboxypeptidases (CNAG_01540 and CNAG_05973), serine-type endopeptidase (CNAG_04625), and elastinolytic metalloprotease (CNAG_04735), was also reduced. Fungal proteases are known to elicit inflammatory responses via activation of protease-activated receptors (PARs), leading to allergic inflammation (63, 64). Thus, reduced secretion of proteases by the Δkcs1 mutant potentially contributes to the weak proinflammatory cytokine response and impaired infiltration of inflammatory neutrophils and monocytes observed in Δkcs1 mutant-infected lung (Fig. 7 and S4).

The persistence of Δkcs1 strain infection also correlated with a reduced recognition of Δkcs1 cells by the host immune system, as indicated by the lung cytokine profile (Fig. 7). First, in contrast to what occurred in WT-infected lung, no peak at day 7 was observed for the TNF, IFN-γ, and MCP-1 responses in Δkcs1 mutant-infected lung. This is consistent with a greater reduction in the proinflammatory response to Δkcs1 cells at day 7 when fungal burden is taken into account. Interestingly, however, similar IL-2 and IL-17A responses were obtained in WT- and Δkcs1-infected lungs at day 14 despite the significantly lower Δkcs1 mutant burden. Taken together, the phagocytosis, lung cytokine, and histological data suggest that the persistence of a low-grade, asymptomatic Δkcs1 mutant infection is attributable to reduced recruitment of monocytes to sites of Δkcs1 mutant infection and reduced uptake of the Δkcs1 mutant by resident lung macrophages and infiltrating monocytes.

In summary, we have demonstrated for the first time that the IP biosynthetic pathway is essential for cryptococcal pathogenicity in a mammalian infection model. We have shown that Kcs1 is the major IP₆ kinase in C. neoformans producing IP₇, while Asp1 is an IP₃ kinase essential for the production of IP₆. We have established that, of the two PP-IP₆, IP₇ exerts the greatest influence on cryptococcal pathogenicity. Our findings indicate that IP₇ affects cryptococcal cellular functions, at least in part, via gene expression and that successful host infection requires IP₇ to activate starvation-related metabolic pathways.

**MATERIALS AND METHODS**

**Strains and media.** Wild-type C. neoformans var. grubii strain H99 (serotype A, MATα) was used in this study. The ARG1 (CNAG_06500) deletion mutant (Δarg1 mutant) was described previously (24). The KN99 (MATα) strain was a kind gift from Joseph Heitman’s laboratory (Duke University, Durham, NC, USA) (65). C. neoformans strains were maintained on SAB agar plates (4% dextrose, 1% peptone, 2% agar, pH 5.6), and incubated in YNB (6.7 g/liter yeast nitrogen base, 0.5% glucose), YPD (1% yeast extract, 2% peptone, and 2% dextrose), or MM (minimal medium; 15 mM glucose, 10 mM MgSO₄, 29.4 mM KH₂PO₄, 13 mM glycine, 3 μM thiamine) broth in preparation for the experiments. Mating was induced on 5% V8 juice (pH 5), 2% agar plates. MM without glucose was used for laccase induction in broth culture and for the preparation of broth and plates with different carbon sources (1% oleic acid, sodium lactate, or glycerol). Minimal medium plates supplemented with 1 mM L-DOPA (1-3,4-dihydroxyphenylalanine) were used to assess melanization.

**Quantification of extracellular laccase activity.** The protocol was adapted from reference 66. C. neoformans strains were grown overnight in YPD at 30°C. Cells were pelleted, washed with water, resuspended in MM without glucose at an optical density at 600 nm (OD₆₀₀) of 1, and incubated for 4 h at 30°C. To measure cell-associated laccase activity, 200 μl of the culture was removed and the cells were pelleted by centrifugation. The pellet was resuspended in 1 ml of a 3 mM concentration of a chromogenic laccase substrate, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), and incubated for 30 min at 30°C until a light blue color was produced, indicative of ABTS oxidation. The cells were pelleted, and the OD of the supernatant was measured at 436 nm. Laccase-specific activity was measured as micromoles of ABTS substrate oxidized/minute/milliliter of culture at an OD₆₀₀ of 1. The concentration of oxidized ABTS was calculated using the molar extinction coefficient (ε = 29.3 M⁻¹ cm⁻¹).

**[3H]inositol labeling.** [3H]inositol labeling of cryptococcal cells was performed according to the protocol of reference 67, with several modifications. Briefly, fungal strains from overnight YPD culture were diluted in 5 ml fresh YPD to an OD₆₀₀ of 0.01 (WT versus the Δkcs1 and Δarg1 mutants) or to an OD₆₀₀ of 0.05 (WT versus the Δkcs1:KCS1, Δkcs1::KCS1_W303, and Δasp1 strains), supplemented with 10 μCi/ml [3H]myo-inositol (PerkinElmer), and incubated until the OD of the cultures reached at least 6 (16 to 76 h). The cells were pelleted, washed twice with 1 ml of fresh YPD, and snap-frozen in liquid nitrogen. To extract inositol polyphosphates, the cells were resuspended in extraction buffer (1 M HClO₄, 3 mM EDTA, 0.1 mg/ml IP₆) and homogenized in the presence of glass beads using a bead beater (four 30-s cycles with 1-min rests in between cycles). After the debris was pelleted, supernatants were neutralized using neutralization buffer (1 M K₂CO₃, 3 mM EDTA) and incubated on ice for 2 h. HPLC inositol phosphate analysis was performed as previously described (67).

**Murine inhalation model of cryptococcosis.** All procedures were approved by the Sydney West Local Health District Animal Ethics Committee, Department of Animal Care. Survival and organ burden cytokine analyses were conducted using 7-week-old female BALB/c mice obtained from the Animal Resource Centre, Floreat Park, Western Australia. For both sets of analyses, mice were anesthetized using isoflurane (in oxygen) delivered via an isoflurane vaporizer attached to a Stinger small-animal anesthetic machine (Advanced Anaesthesia Specialists). Groups of 8 to 10 mice were then inoculated intranasally with the C. neoformans WT or IPK mutant strain (5 × 10⁶ yeast cells in 20 μl phosphate-buffered saline [PBS]) and observed daily for signs of ill health. The number of viable yeast cells inoculated into the nares was later confirmed by quantitative culture.

For the survival study, mice which had lost 20% of their preinfection weight or which showed debilitating clinical signs prior to losing this weight or which showed debilitating clinical signs prior to losing this...
weight, including hunching, respiratory distress, excessive fur ruffling, or sluggish/unsteady movement, were euthanized by CO2 inhalation followed by cervical dislocation. \( \Delta kcs1 \) mutant-infected mice were sacrificed 50 days postinoculation. Differences in survival were determined with SPSS version 21 statistical software, using the Kaplan-Meier method (log rank test), where a \( P \) value of <0.05 was considered statistically significant.

For calculating lung and brain infection burdens and determining lung cytokine profiles, 3 from a group of 9 mice infected with a particular strain (the WT or \( \Delta kcs1 \) mutant) were sacrificed 3, 7, and 14 days postinoculation. For lung cytokine analysis, 3 mock-infected mice sacrificed on days 3, 7, and 14, respectively, served as controls. For determining infection burdens, lungs and brains were harvested from infected mice, weighed, and homogenized in a total of 1 ml sterile PBS. One hundred microliters of each homogenate were removed and serially diluted 10-fold in sterile PBS, and 100 \( \mu l \) of each dilution was plated onto SAB agar. The plates were incubated for 2 to 3 days at 30°C, and the number of CFU was determined. The remainder of each lung homogenate (900 \( \mu l \)) and 900 \( \mu l \) of lung homogenate obtained from each of the control mice were centrifuged at 10,000 \( \times g \) to pellet tissue debris, and the supernatants were collected and frozen for cytokine analysis.

**Cytokine profiling in infected lung supernatant.** Cytokine concentrations in lung homogenates collected postinfection were determined using mouse Th1/Th2/Th17 cytokine bead arrays (CBA; Becton, Dickinson Biosciences). MCP-1 was measured in a separate assay using the mouse MCP-1 flex set (bead B7) (BD Biosciences). CBA kits were used according to the manufacturer’s instructions, data were collected using an LSRII flow cytometer, and results were analyzed using FCAP software version 3.0 (BD Biosciences).

**Virulence in Galleria mellonella.** *C. neoformans* WT and \( \Delta kcs1 \) mutant cells were grown overnight in YPD broth, pelleted by centrifugation, and resuspended in water at a concentration of 10\(^6\) cells/ml. *G. mellonella* larvae (10 per strain) were inoculated with 10 \( \mu l \) of cell suspension (10\(^6\) yeast cells) by injection into the hemocoel via the lower prolegs. The viability of each inoculum was assessed by performing serial 10-fold dilutions, plating the lowest dilutions on SAB agar plates, and counting the CFU after a 3-day incubation at 30°C. Inoculated larvae were monitored daily for 10 days after daily removal of their cocoons. Larvae were considered dead when they no longer responded to touch (68).

**Phagocytosis experiments.** THP-1 cells were maintained in RPMI 1640-10% fetal bovine serum (FBS) medium. IFN-\( \gamma \) (10 ng/ml) was added 24 h prior to the phagocytosis experiment to activate the monocytes. Cryptococcal cells were grown overnight in YNB broth, pelleted by centrifugation, and resuspended in staining solution (0.5 mg/ml FITC in PBS) for 30 min at 30°C. To heat kill fungal cells in some of the experiments, YNB cultures were centrifuged and the cell pellets were incubated at 56°C for 30 min prior to FITC staining. Following FITC staining, fungal cell pellets were washed three times with PBS and opsonized for 30 min at 30°C with human serum (10 to 100%, as indicated in the figures) or human IgG (Sigma-Aldrich 14506) or anti-GXM antibody 18B7 (a kind gift from Arturo Casadevall) in PBS (69). Opsonized fungal cells were pelleted and mixed with THP-1 monocytes in a ratio of 3:1 or 5:1 in THP-1 growth medium. Fungal and mammalian cells were coincubated for 3 to 5 h at 37°C in 5% CO\(_2\). To quantify the adherence/uptake of the fungal cells by THP-1, the cultures were mixed by pipetting, diluted with PBS, and analyzed by flow cytometry and microscopy. Reverse transcription-PCR (RT-PCR) was used to assess the THP-1 activation response to WT and \( \Delta kcs1 \) cells after 4 h of coincubation, using an RT2 profiler PCR array designed for analysis of the antifungal immune response (SABiosciences). PCR was performed using a QuantStudio® Flex real-time PCR system (Life Technologies). The expression data were normalized to the expression of the housekeeping GAPDH, ACTB, B2M, Hprt1, and Rplp0 genes and then calculated as the fold change from expression in activated THP-1 cells that had not been coincubated with cryptococci.

Peripheral blood monocytes (PBMCs) were isolated from fresh human blood using a Ficoll-Paque gradient. Up to 20 ml of blood was layered on top of 15 ml of Ficoll-Paque and centrifuged at 400 \( \times g \) for 30 min. The interphase containing monocytes and T and B lymphocytes was collected in a fresh tube and washed twice with PBS before resuspension of the final pellet in RPMI 1640-10% FBS medium. PBMCs were visualized with crystal violet stain and counted using a hemocytometer. PBMCs (4 \( \times 10^6 \)) were mixed with 4 \( \times 10^6 \) heat-killed, FITC-labeled, 10% serum-opsinnized cryptococci in 100 \( \mu l \) of RPMI 1640-10% FBS medium and coincubated for 2 h at 37°C in 5% CO\(_2\). The cell pellets were washed once with PBS. Nonspecific Fc receptor-mediated antibody binding was blocked before cells were stained with antigen-presenting cell (APC)-H7-conjugated anti-CD14 antibody (monocyte marker) (BD Biosciences; catalog number 560180). Adhesion/uptake of cryptococci by the CD14-positive cell population was quantified by flow cytometry and visualized by microscopy (70, 71; see Text S1 in the supplemental material).

**mannoprotein staining with ConA-FITC.** Cryptococcal cells were grown overnight in YNB, washed with PBS, and resuspended in 100 \( \mu l \) PBS supplemented with 0.5% gelatin at an OD\(_{500}\) of 0.5. Each suspension was incubated for 30 min at room temperature. Ten microliters of ConA-FITC (Sigma; C7642) from a 2.5-mg/ml stock solution in water was added to each culture sample, followed by incubation for 30 min at room temperature. The extent of ConA-FITC binding was assessed using flow cytometry.

**High-throughput gene expression analysis.** Fungal strains were grown in YPD broth for 22 h. RNA was isolated using TRIzol and further purified using a Qiagen RNA isolation kit. Library preparation and sequencing were carried out by the Ramaciotti Centre for Genomics (UNSW, Sydney, Australia) using a TrueSeq sample preparation kit and the Illumina HiSeq2000 sequencing platform to generate 100-bp paired-end reads. The resulting data were analyzed using the Galaxy platform (https://usegalaxy.org/). The analysis included data from three biological replicates for each strain; a \( \geq 2 \)-fold difference in gene expression between WT and \( \Delta kcs1 \) cells was considered to be significant. The presence of a signal peptide and N- and O-linked glycosylation were predicted using CBS prediction tools (http://www.cbs.dtu.dk/services/). The presence of a GPI anchor was predicted using the Big-PI fungal predictor (http://mdel.imp.ac.at/sat/gpi/fungi_server.html). Initial functional classification of cryptococcal genes was performed using GO term assignment from the Joint Genome Institute (JGI) database (http://genome.jgi.doe.gov/cgi-bin/ToGo?species=Crane_ H99_1) and further improved based on the literature and by performing a homology search. A hypergeometric test was used to assess whether Kcs1-dependent genes were overrepresented in specific functional groups.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00531-15/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 1 MB.
Figure S2, TIF file, 0.4 MB.
Figure S3, TIF file, 1.4 MB.
Figure S4, TIF file, 1.9 MB.
Figure S5, TIF file, 0.2 MB.
Figure S6, TIF file, 0.5 MB.
Table S1, DOC file, 0.1 MB.
Table S2, DOC file, 0.1 MB.
Table S3, PDF file, 0.1 MB.

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