Phospholipase C (PLC) of Cryptococcus neoformans (CnPlc1) is crucial for virulence of this fungal pathogen. To investigate the mechanism of CnPlc1-mediated signaling, we established that phosphatidylinositol 4,5-bisphosphate (PIP2) is a major CnPlc1 substrate, which is hydrolyzed to produce inositol trisphosphate (IP3). In Saccharomyces cerevisiae, Plc1-derived IP3 is a substrate for the inositol polyphosphate kinase Arg82, which converts IP3 to more complex inositol polyphosphates. In this study, we show that in C. neoformans, the enzyme encoded by ARG1 is the major IP3 kinase, and we further demonstrate that catalytic activity of Arg1 is essential for cellular homeostasis and virulence in the Galleria mellonella infection model. IP3 content was reduced in the CnΔplc1 mutant and markedly increased in the CnΔarg1 mutant, while IP2 was increased in both mutants. The CnΔplc1 and CnΔarg1 mutants shared significant phenotypic similarity, including impaired thermotolerance, compromised cell walls, reduced capsule production and melanization, defective cell separation, and the inability to form mating filaments. In contrast to the S. cerevisiae ARG82 deletion mutant (ScΔarg82) strain, the CnΔarg1 mutant exhibited dramatically enlarged vacuoles indicative of excessive vacuolar fusion. In mammalian cells, PLC-derived IP3 causes Ca2+ release and calcineurin activation. Our data show that, unlike mammalian PLCs, CnPlc1 does not contribute significantly to calcineurin activation. Collectively, our findings provide the first evidence that the inositol polyphosphate anabolic pathway is essential for virulence of C. neoformans and further show that production of IP3 as a precursor for synthesis of more complex inositol polyphosphates is the key biochemical function of CnPlc1.

Cryptococcus neoformans var. grubii is a human fungal pathogen that predominantly infects immunocompromised hosts. After inhalation from the environment, infection spreads from the lung to the brain via the bloodstream, causing life-threatening meningocencephalitis. Several factors contribute to the virulence of C. neoformans, including the ability to grow at physiological temperature (37°C) and to produce melanin and capsule (1–3). Our previous work demonstrated that these virulence determinants are attenuated by deletion of a single gene encoding a key signaling molecule, phosphatidylinositol (PI)-specific phospholipase C (PLC) (Plc1). In addition, the C. neoformans PLC1 deletion mutant (CnΔplc1) had a defective cell wall and compromised virulence in both mouse (37°C) and Caenorhabditis elegans (25°C) models of cryptococcosis (4). A second PLC homolog, Plc2, appears to have no significant role in the biology and virulence of C. neoformans (4).

In mammalian cells, PI-specific phospholipase C enzymes (PLCs) catalyze the hydrolysis of PI-(4,5)-bisphosphate (PIP2) to diacylglycerol (DAG) and D-myo-inositol-(1,4,5)-trisphosphate (IP3). DAG directly activates protein kinase C (PKC), while IP3 triggers release of calcium from intracellular stores via interaction with IP3 receptors in the endoplasmic reticulum (ER). Elevated cytosolic calcium activates calcium-dependent signaling enzymes, such as calcineurin. In the model yeast Saccharomyces cerevisiae, a single PLC isoform has similar catalytic activity to the mammalian PLC-δ isofrom (5). However, the role of the IP3 breakdown products and the mechanism of Plc1-dependent signaling remain to be fully elucidated. Similarly, in C. neoformans, the identity and fate of the CnPlc1 hydrolysis products have not been determined.

In fungi, the role of IP3-derived IP2 in initiating calcium influx is controversial as no homologs of mammalian IP3 receptors have been identified in fungal genomes. In the filamentous fungus Neurospora crassa, the existence of a functional IP3 receptor equivalent has been inferred on the basis of an IP3-dependent increase in cytoplasmic Ca2+. N. crassa PLC is thought to be activated by membrane stretch, generating IP3, which triggers Ca2+ release from hyphal tip vesicles, thus maintaining a high Ca2+ concentration required for hyphal tip growth (6). In S. cerevisiae, however, transient IP3 increase in nitrogen-depleted cells following addition of ammonium sulfate did not trigger a spike in intracellular calcium (7). In glucose-starved S. cerevisiae, glucose addition...
caused PLC-dependent influx of extracellular calcium accompanied by a transient increase in IP$_3$. However, it is unclear as to whether calcium influx is triggered by IP$_3$ or via direct interaction of PLC with a plasma membrane calcium channel (8, 9).

In fungi, increased cytosolic calcium triggered by stress or morphological changes activates the protein phosphatase calcineurin via calcium-bound calmodulin. In C. neoformans, calcineurin responds to stress caused by cell-wall-perturbing agents, host physiological temperature, high CO$_2$, alkaline pH, and high cation concentration. Moreover, calcineurin is essential for virulence and hyphal elongation during mating and monokaryotic fruiting (10, 11). In S. cerevisiae, calcineurin conducts most of its transcriptional regulation via activation of the zinc finger transcription factor Crz1. In C. neoformans, the Crz1 ortholog is also activated by calcineurin and, during starvation, by protein kinase C, to regulate cell wall integrity (12, 13).

An alternative fate of PLC-generated IP$_3$ in S. cerevisiae is phosphorylation by inositol polyphosphate kinases (IPKs). The resulting inositol polyphosphates (IPs) influence chromatin remodeling, transcriptional regulation, mRNA export, telomere length, vacuole morphogenesis, endocytosis, and cell division via an effect on kinetochore activity (14; for review, see reference 15). In S. cerevisiae, the single IPK Arg82 converts Plc1-derived IP$_3$ into IP$_4$ and IP$_5$, which can be further phosphorylated to IP$_6$ by Ipk1. Kcs1 and Vip1 then convert IP$_6$ to different isoforms of the inositol pyrophosphate (PP-IP) PP-IP$_5$. PP-IPs contain two phosphates on the same position of the inositol ring. Arg82 and Kcs1 can also use IP$_3$ as a substrate to generate two isoforms of PP-IP$_4$ (16). Similar to deletion of PLC1 in C. neoformans, deletion of ARG82 in S. cerevisiae results in a pleiotropic phenotype. The S. cerevisiae ARG82 deletion mutant (ScΔarg82) abnormalities include temperature sensitivity, sterility, and defective sporulation. We therefore hypothesized that the key role of Plc1 in C. neoformans is to produce IP$_3$ as a precursor for the synthesis of more complex IPs and that the deletion of the ARG82 ortholog in C. neoformans would produce a pleiotropic phenotype similar to that of the CnΔplc1 mutant due to the lack of IP$_4$–IP$_8$ species in both mutants.

In this study, we investigated the biochemical mechanism underlying Plc1-mediated signaling in C. neoformans. Our findings suggest that the main function of cryptococcal Plc1 is to supply IP$_3$ as a substrate for an inositol polyphosphate kinase, Arg1, and that the catalytic activity of Arg1 is essential for cellular homeostasis and virulence of C. neoformans.

**MATERIALS AND METHODS**

**Strains and media.** Wild-type (WT) C. neoformans var. grubii strain H99 (serotype A, MATa) was used in this study. The Δnal1 mutant and the KN99 (MATb) strain were a kind gift from Joseph Heitman’s laboratory (Duke University, Durham, NC). The Δmpk1 mutant was also a gift from Jenny Lodge’s laboratory (Washington University, St. Louis, MO). C. neoformans strains were routinely cultured on YPD medium (1% yeast extract, 2% peptone, 2% dextrose). Mating was induced on 5% V8 juice (pH 5)–2% agar plates. Minimal medium (MM) (15 mM glucose, 10 mM MgSO$_4$, 29.4 mM KH$_2$PO$_4$, 13 mM glycine, 3 μM thiamine) was used for capsule induction. MM agar plates supplemented with 1 mM L-DOPA (1,3,4-dihydroxyphenylalanine) were used to assess melanization.

**CnPlc1 expression in S. cerevisiae.** The C. neoformans PLC1 (CNAG_02867) coding sequence (see Table 1 for primer sequences) was amplified from a C. neoformans cDNA library (a gift from Peter Williamson, NIH), cloned into the pYES2/NT vector (Life Technologies), and transfected into the S. cerevisiae ARG82 deletion mutant (ScΔarg82) by electrotransformation. Cells were plated on minimal media containing 2% raffinose and incubated on a shaker at 30°C. Transformed cells were verified by PCR and positive transformants were selected by their ability to grow on MM media. The transformed cells were further verified by Western blotting using a mouse monoclonal antibody directed against the C. neoformans glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein.

**TABLE 1** Primers used in this study

<table>
<thead>
<tr>
<th>Primer$^a$</th>
<th>Description</th>
<th>Sense Sequence</th>
<th>Antisense Sequence</th>
</tr>
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<tr>
<td>C. neoformans PLC1 BamHI-s</td>
<td>Verification of C. neoformans PLC1 expression</td>
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<tr>
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<tr>
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<td>ARG1-2-a</td>
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<tr>
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<td>Internal ARG2 deletion construct and rec gDNA fragment</td>
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<td>ACT1-a</td>
</tr>
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$^a$ The lowercase letters represent sequence with no homology to template DNA, whereas homologous regions are shown by uppercase letters.
transformed into S. cerevisiae strain INVSc1. Expression of CnPLC1 was induced by growing the cells in synthetic defined (SD) medium without uracil, replacing glucose with galactose as the sole carbon source. For Plc1 protein purification, yeast cells were processed as previously described (5). In brief, the cells were lysed in buffer A (50 mM Tris [pH 7.9], 400 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], protease inhibitor cocktail) by vigorous vortexing with 0.5-mm glass beads. The protein extracts were clarified by centrifugation and subjected to Ni²⁺-affinity chromatography. The eluted fractions that were demonstrated to contain Plc1 protein were combined and dialyzed against buffer B (25 mM HEPES-HCl [pH 7.2], 50 mM NaCl, 1 mM EDTA).

**PLC activity assay.** The PLC-specific activity of purified CnPlc1 and the crude protein extracts was determined using a radiometric enzyme assay, with PI or PIP₂ as the substrate, as described previously (5) with modifications. For the PIP₂ hydrolysis assay, the substrate was prepared by combining 10 nmol of cold 1-α-phosphatidylinositol 4,5-diphosphate (Sigma; P9763) dissolved in 20 µl chloroform-methanol-water–1 N HCl (20:10:1:1 [vol/vol]) with 1.5 µL (0.015 µC) of hot phosphatidylinositol-4,5-bisphosphate ([inositol-2-³H][N]) (Perkin-Elmer; NET895005UC) per reaction. PIP₂ substrate mix was dried under nitrogen and resuspended in 25 µl of 2X PIPLC-PIP₂ assay buffer (PPAB) (1.6 mM Triton X-100, 100 mM HEPES [pH 7.0], 200 mM NaCl, 2 mM EGTA, 2 mM CaCl₂) per substrate. Substrate dissolution was aided by sonication in a water bath sonicator (Soniclean, South Australia, Australia) for 10 min with occasional vortexing. Each assay was initiated by the addition of 25 µl of substrate-PPAB to 5 to 25 µg protein. The final volume of the reaction mixture was adjusted to 50 µl. Incubation was carried out at 30°C for 15 min. The tubes were placed on ice, and 100 µl of 1% bovine serum albumin (BSA) was added to each tube followed by 250 µl of 10% trichloroacetic acid (TCA). The tubes were centrifuged, and the ³H radioactivity in the supernatant (100 µl/reaction) following the manufacturer's instructions. To quantify PIP₂, the insoluble pellet obtained by centrifugation of the homogenized cells was washed with 1.5 ml of ice-cold water, resuspended in 940 µl chloroform-methanol-HCl (80:40:1 [vol/vol]), and vortexed for 15 min. The lipid and aqueous phases were resolved by adding 310 µl chloroform and 560 µl 0.1 M HCl, vortexing, and centrifugation at 1,000 × g for 15 min at room temperature. Four hundred microliters of the lower phase was dried under a stream of nitrogen. The dried samples were dissolved in 250 µl of 1 M KOH and heated at 100°C for 15 min to hydrolyze PIP₂. After cooling on ice, the samples were supplemented with 15 µl HEPES (1 M [pH 7.2]) and further neutralized to pH 7.5 by titration with ice-cold 4% perchloric acid. The samples were centrifuged at 2,000 × g for 15 min at 4°C to remove KC1Ο₄ sediments. The IP₃ produced as a result of PIP₂ hydrolysis (100 µl of supernatant/reaction) was quantified using the inositol-1,4,5-triphosphate [³H] radioreceptor assay kit (PerkinElmer Life Sciences).

**Generation of transgenic strains.** Plasmid pIAF (containing a neomycin resistance [NEO<sup>®</sup>] cassette) was kindly provided by John R. Perfect, Duke University, Durham, NC. The ARG1 and ARG2 gene deletion constructs were made by overlap PCR, joining the 5’ flank, NEO<sup>®</sup> from pIAF (ACT1 promoter, neomycin phosphotransferase, TRP1 terminator) and the 3’ flank (see Fig. S1 in the supplemental material) with the 3’ of the gene of interest. Transformation was carried out by the biolistic method (17). NEO-resistant transformants were selected on YPD agar plates supplemented with 0.5 M sorbitol and 100 µg/ml G418. Correctly targeted integration of the ARG1 and ARG2 deletion constructs was confirmed by PCR amplification across the junction point of integration of the construct with genomic DNA (gDNA), using a forward primer that anneals outside the region of integration and a reverse primer that anneals within the construct (see Fig. S1 in the supplemental material). The Δarg1: ARG1 1-reconstituted strain was created by transforming the Δarg1 mutant with a genomic fragment of ARG1 that included 1,169 bp upstream and 1,133 bp downstream of the coding region (primers 5’-5’-3’). To select for ARG1-expressing colonies, transformed cells were plated on YPD agar supplemented with 0.5 M sorbitol and 0.005% Congo red and incubated at 37°C. To confirm that the colonies which grew under these conditions were true Δarg1:ARG1 reconstituted strains, the cells were tested for the presence of both ectopically integrated ARG1 and the ARG1 deletion cassette by PCR (see Fig. S1).

**Virulence in Galleria mellonella.** C. neoformans WT and mutant strains were grown overnight, pelleted by centrifugation, and resuspended in phosphate-buffered saline (PBS) at a concentration of 10⁶ cells/ml. G. mellonella larvae (10 per strain) were inoculated with 10 µl of cell suspension (10⁴ yeast cells) by injection into the hemocoel via the lower pro-legs. The viability of each inoculum was assessed by performing serial 10-fold dilutions, plating the lowest dilutions on Sabouraud agar (SAB) plates, and counting the CFU after 3 days of incubation at 30°C. Inoculated larvae were monitored daily for 10 days. The Kaplan–Meier method in the SPSS version 21 statistical software was used to estimate the differences in survival (log rank test) and to plot the survival curves. In all cases, a P value of <0.05 was considered statistically significant. The number of viable fungal cells in larvae was determined by homogenizing each larva in 1 ml PBS and preparing and plating serial dilutions of each homogenate as described for the inoculum.
Gene expression. To compare levels of chitin synthase CHS6 (CNAG_00546) expression, the cells were cultured in YPD overnight at 30°C and diluted to an OD of 0.5. After 4 h of incubation, the cells were treated with calcofluor white (CFW) (2.2 mg/ml) with or without FK506 (10 μg/ml) for 1 h. The cells were collected and snap-frozen. RNA extraction and cDNA synthesis were performed as described previously (12). Real-time PCR was performed using the actin-encoding gene (ACT1) for normalization.

Microscopy. For staining of C. neoformans vacuoles, the cells were grown in YPD overnight, diluted 1:10 in fresh medium, and incubated for 4 h. The cells were pelleted and resuspended in 50 mM sodium citrate buffer (pH 5), supplemented with 1 μM carboxy-DCFDA (carboxy-5-(and-6)-chloromethyl-2',7-dichlorodihydrofluorescein diacetate) (Life Technologies), and incubated for 20 min. Measurements of vacuolar size were performed using ImageJ software (NIH). For the FM-4-64 pulse-chase experiment, the YPD-grown cells were loaded with 10 μM FM-4-64 in YPD for 5 min (time point 0), pelleted, and resuspended in fresh YPD medium. The tubes were incubated at 30°C with shaking. Aliquots were withdrawn at the indicated times, and the cells were pelleted and kept on ice until observed by microscopy.

For F-actin staining, the cells were grown overnight in YPD, pelleted and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were washed twice with PBS and permeabilized with 1% Triton X-100 in PBS for 8 min. Following permeabilization, the cells were washed twice with PBS and incubated with 1% BSA in PBS for 20 min, followed by staining with Alexa Fluor 488-conjugated phalloidin in PBS (Life Technologies; 25 U/100 μl cells) for 90 min. DAPI (4′,6-diamidino-2-phenylindole; 1 μg/ml) was added for the final 3 min of the 90-min incubation with Alexa Fluor 488-conjugated phalloidin.

RESULTS
CnPlc1 preferentially hydrolyzes PIP2. To establish that Plc1 is enzymatically active and to determine its substrate preference, we expressed recombinant His-tagged CnPLC1 in S. cerevisiae under the regulation of a galactose-inducible promoter (Fig. 1A). The enzyme was then purified by nickel affinity chromatography. Purified enzyme and crude lysates were assayed for hydrolytic activity against PI or PIP2, and release of 3H-inositol from each substrate was measured by β-counting. Results represent means ± standard errors (SE) (n ≥ 3). *, statistically significant difference relative to Sacch-CnPlc1 lysate and nickel affinity (His)-purified Plc1 (P < 0.001, using a two-tailed t test). #, statistically significant difference relative to nickel affinity (His)-purified Plc1 (P = 0.01).

Identification of the major inositol polyphosphate kinase (Arg1) in C. neoformans. In S. cerevisiae, PLC-derived IP3 is converted to IP2 and IP4 by the inositol polyphosphate multikinase Arg82 (16, 18). Using the S. cerevisiae protein in a similarity search, we identified two Arg82 homologs in the C. neoformans genome: CnArg1 (CNAG_06500; NCBI accession no. AFR98730) and CnArg2 (CNAG_02802; NCBI accession AFR93890), which share 22% and 15% identity, respectively, with ScArg82, and 17% identity with each other (see Fig. S2 in the supplemental material).
of IP_3 in the CnΔarg1 mutant establishes CnArg1 as the major IP_3 kinase in *C. neoformans*.

In addition to reduced IP_3 content, deletion of *PLC1* potentially leads to accumulation of its PIP_2 substrate, with a resultant deleterious effect on yeast cells (21). We therefore measured the PIP_2 membrane content in Δ*plc1* and Δ*arg1* cells indirectly, by measuring IP_3 produced by alkaline hydrolysis of PIP_2 (Fig. 2B). Surprisingly, PIP_2 accumulation was even more pronounced in Δ*arg1* cells than in Δ*plc1* cells. This finding implies product inhibition of Plc1 activity in Δ*arg1* mutants by the high concentration of IP_3.

**Arg1 is essential for virulence, cell wall integrity, and mating in *C. neoformans.* Changes in IP_3 content in Δ*plc1* and Δ*arg1* cells (Fig. 2A) demonstrate that Plc1 and Arg1 function within the same signaling pathway, with Plc1 providing a substrate (IP_3) for Arg1. Arg1 catalytic products and their derivatives are expected to be absent in both mutants, as is the case in *S. cerevisiae* (22, 23). To assess the role of the Arg1 protein and its products in virulence and homeostasis, we characterized the Δ*arg1* phenotype, as compared to the Δ*plc1* mutant and the wild-type strain.

Similar to the Δ*plc1* mutant, growth of the Δ*arg1* mutant was delayed under normal growth conditions (30°C, YPD). However, growth of both strains was markedly inhibited at human physiological temperature (37°C) and in the presence of the cell-wall-perturbing agent Congo red (Fig. 3A). Melanization on minimal medium containing l-DOPA (Fig. 3B) and the formation of mating filaments in a unilateral cross with KN99 (MATa) (Fig. 3C) were also reduced. In the case of temperature stress and mating, the phenotype of the Δ*arg1*:Δ*ARG1* strain was completely restored to that of the WT. However, in the case of Congo red sensitivity and melanization, the phenotype of the Δ*arg1*:Δ*ARG1* strain was intermediate between those of the WT and Δ*arg1* strains, consistent with partial restoration of the Δ*arg1* phenotype by the ectopically integrated *ARG1*. Both the Δ*plc1* and Δ*arg1* mutants formed smaller capsules than the WT cells under capsule-inducing conditions, with capsule size restored to that of WT in the Δ*arg1*:Δ*ARG1* strain (Fig. 3D). Unlike the Δ*arg1* mutant, the Δ*arg2* strain exhibited WT-like tolerance to elevated temperature and cell wall stress (see Fig. S3B in the supplemental material). The Δ*arg2* strain also produced melanin and capsules similar to the WT strain (see Fig. S3C and D). The lack of phenotypic defects in the Δ*arg2* strain is consistent with unaltered IP_3 content in this mutant (see Fig. S3A).

The virulence of the Δ*arg1* and Δ*plc1* mutants was compared with that of the WT in larvae of the greater wax moth, *Galleria mellonella*. The *G. mellonella* infection model was chosen over the mouse inhalational model as larvae can be maintained at the permissive growth temperature of the mutant strains (30°C). At the mouse body temperature (∼37°C), the growth of both mutants was severely compromised (Fig. 3A). Larvae were inoculated with 1 × 10^6^ cryptococci. The inoculum was plated out to quantify the inoculum as CFU. These were determined to be 0.8 × 10^6^, 0.5 × 10^6^, and 1.1 × 10^6^ CFU for the WT, Δ*plc1* mutant, and Δ*arg1* mutant, respectively. While all of the larvae inoculated with WT *C. neoformans* died within 3 days, Δ*arg1* strain-inoculated insects died more slowly (median survival of ∼5 days) (Fig. 4). Unlike the Δ*arg1* mutant, infection with the Δ*plc1* strain caused the death of only 1 larva out of 10 over the entire infection period. Survival of the larvae was analyzed using the Kaplan-Meier method (log rank test). Overall, the difference in survival rates was statistically significant (P < 0.001). For the pairwise comparisons, the survival
rates of PBS-treated and Δplc1 strain-infected larvae were comparable (P = 0.317). Survival of PBS-treated and Δplc1 strain-infected larvae was significantly greater than that of Δarg1 strain-infected larvae (P = 0.001), which in turn was significantly greater than that of the WT-infected larvae (P < 0.001).

Fungal cells were retrieved from infected insects and quantified. WT cells had propagated dramatically within larvae that had succumbed to infection on day 3 postinoculation, with ~128-fold more CFU recovered compared with the number inoculated. The Δarg1 cells also propagated within larvae, but not to the same extent as the WT, with ~25-fold more CFU recovered from larvae at death on day 4 postinoculation. Interestingly, the Δplc1 cells did not propagate in larvae, as similar CFU to those inoculated were recovered from healthy larvae on day 6 postinoculation.

**Altered vacuolar morphology, cell separation, and endocytosis in Δplc1 and Δarg1 mutants.** We previously demonstrated that Δplc1 mutant cells had irregular morphology and contained enlarged vacuoles, compared with the WT strain (4). Similar to the Δplc1 cells, Δarg1 cells appeared to have enlarged vacuoles (Fig. 3D). To further investigate vacuolar morphology, vacuolar lumen were stained with carboxy-DCFDA and vacuole size and number per cell were documented (Fig. 5A and B). Cells of all strains contained numerous small vacuoles. However, Δarg1 cells often contained one markedly enlarged vacuole, implying excessive vacuolar fusion in this mutant (Fig. 5A, arrowheads). The average vacuole size in Δarg1 cells (2.2 μm) is 3-fold larger than that in WT cells (0.63 μm), while the vacuoles of Δplc1 cells (average size, 0.97 μm) are only slightly larger than the vacuoles of the WT (Fig. 5B). In all strains, the number of vacuoles per cell inversely correlated with vacuole size. On average, WT cells contained 3.5 vacuoles, compared to 1.3 and 2 vacuoles in Δarg1 and Δplc1 cells, respectively (Fig. 5B).

Δplc1 and Δarg1 cells often formed aggregates of three or four conjoined cells (Fig. 5C, broken arrows), while WT cells were only joined in pairs. Since defects in morphology and cell separation might reflect abnormal polarized growth, WT, Δplc1, and Δarg1 cells were stained with Alexa Fluor 488-conjugated phalloidin to visualize F-actin distribution. In all strains, actin patches were distributed throughout the cytosol of mature cells and accumulated in the emerging buds, as described previously (24). In the WT, most of the conjoined cells were premitotic, with nuclei visible only in the mother cell. However, in the Δplc1 and Δarg1 mutants, cells often failed to separate after mitosis, and nuclei were visible in both mother and daughter cells. Furthermore, brightly stained actomyosin rings were often observed at the junctions between these conjoined mutant cells (Fig. 5C, solid arrows). These actomyosin rings eventually disappeared, and the actin patches became redistributed throughout the conjoined cells. Despite being joined, these cells sometimes formed new buds. Acto-
myosin rings were rarely observed in WT cells. A similar cell separation defect accompanied by increased localization of F-actin in the medial ring was observed in Schizosaccharomyces pombe deficient in the IP5 2-kinase, Ipk1 (25).

In S. cerevisiae, inositol pyrophosphates regulate endocytic trafficking (23). As we expected inositol pyrophosphates to be absent from the CnΔplc1 and CnΔarg1 mutants, we investigated the dynamics of endocytosis in these mutants by tracking internalization of the lipophilic dye FM4-64 (Fig. 6). The cells were labeled with the dye for 5 min and then chased with fresh medium for different time periods to follow the progress of endocytosis. After a 10-min chase, FM4-64 staining in WT cells was visible in small vesicles and endosomal and, possibly, vacuolar membranes, while in Δarg1 cells, FM4-64 had reached the membranes of large vacuoles, a hallmark of this mutant (Fig. 5). In most Δplc1 cells, FM4-64 was still associated with the plasma membrane after a 10-min chase. Between 10 and 60 min, the FM4-64 staining patterns of WT and Δarg1 cells remained largely unchanged. In the Δplc1 mutant, the dye became visible inside the cells after a 40-min chase, and by 60 min had stained the endosomal and vacuolar membranes (Fig. 6).

CnPlc1 does not contribute significantly to calcineurin activation. In mammalian cells, PLC1 is a well-established regulator of IP3-mediated calcium release into the cytosol and cytosolic calcium activates calcineurin. In C. neoformans, the Δplc1 mutant shares several phenotypic characteristics with the Δcna1 calcineurin deletion mutant: enlarged cells at 30°C, sensitivity to Congo red, growth retardation at 37°C, and a mating defect (Fig. 3)(4, 10, 12). However, there are also significant phenotypic differences between these mutants: only the Δcna1 mutant is sensitive to calcofluor white (CFW) (see below) and has enlarged capsules (our unpublished observation).

To determine whether Plc1 is essential for calcineurin activation, we investigated whether the Δplc1 mutant defects, which are
common to the Δcna1 strain, could be rescued by supplying exogenous calcium to the Δplc1 growth medium. Our previous findings established that, similar to other fungal species, C. neoformans calcineurin is activated by the addition of exogenous calcium to the growth medium (12). Furthermore, in filamentous fungi, addition of calcium restored some of the defects observed in the Δplc1 mutant, such as growth rate in Cryphonectria parasitica, and formation of infection structures (appressoria) in Magnaporthe oryzae (26, 27). In S. cerevisiae, addition of calcium to the medium allowed the Δplc1 mutant to grow at 38°C (28). However, addition of calcium to the C. neoformans Δplc1 mutant (50 mM or 5 mM CaCl₂) did not restore mating ability, growth at 37°C, or resistance to cell wall stress, indicating that impaired calcineurin activation in the absence of Plc1 is not the cause of the defects observed in this mutant (Fig. 7).

We previously demonstrated that the expression of the chitin synthase-encoding gene, CHS6, is upregulated in response to cell wall perturbation with CFW and that CHS6 expression requires functional calcineurin (12). To assess the activity of calcineurin in the absence of Plc1, we tested CHS6 expression in the Δplc1 mutant, compared to that in the WT and Δcna1 strains. CHS6 expression was reduced ~2-fold in the Δplc1 mutant, compared to the WT. However, upon addition of the calcineurin inhibitor FK506, CHS6 expression dropped by ~12- and 25-fold in the WT and Δplc1 strains, respectively. The reduction of CHS6 expression in the Δplc1 strain indicates that calcineurin is active in this mutant (Fig. 8A).

Furthermore, growth of the Δplc1 mutant was inhibited in the presence of FK506 at 30°C, while the Δcna1 mutant grew normally, indicating that calcineurin is essential at this growth temperature in the absence of Plc1 (Fig. 8B). Moreover, calcineurin inhibition caused the Δplc1 mutant to become more sensitive to CFW than the Δcna1 mutant. In C. neoformans, protein kinase C regulates cell wall integrity via activation of the mitogen-activated protein (MAP) kinase cascade, in which Mpk1 is a terminal kinase (29, 30). This pathway functions independently of calcineurin, although cross talk between the two pathways has been observed (30, 31). Similar to the Δplc1 mutant, growth of the cell wall integrity pathway mutants (Δpkc1 and Δmpk1) was inhibited by FK506 in the presence and absence of CFW. These findings suggest that Plc1, calcineurin, and the Pkc1/Mpk1 cell wall integrity
pathway function in parallel to maintain cell wall integrity. Although we cannot rule out the possibility of cross talk between the Plc1 and calcineurin pathways, collectively our results suggest it is unlikely that Plc1 contributes significantly to calcineurin activation in C. neoformans.

**DISCUSSION**

In this report, we present for the first time evidence that metabolism of inositol polyphosphates is essential for the phenotypic expression of virulence determinants in human fungal pathogens. We have studied this pathway in the basidiomycete *C. neoformans* and show that the main biochemical and cellular role of Plc1 is to produce IP3 as a substrate for the inositol polyphosphate kinase Arg1. We have shown that Plc1 and Arg1, and therefore the inositol polyphosphate anabolic pathway, are key regulators of several important virulence attributes of *C. neoformans*, including cell wall integrity, growth at elevated temperature, melanin production, and capsule biosynthesis, suggesting a broad role for inositol polyphosphates in cellular homeostasis, growth, and pathogenicity of *C. neoformans*. Two further important findings from our study are that, unlike in mammalian cells, CnPlc1-derived IP3 does not contribute significantly to calcineurin activation and that IP3 homeostasis in *C. neoformans* regulates cellular function differently from that in the nonpathogenic model yeast, *S. cerevisiae*.

We have demonstrated that CnPlc1 has a similar catalytic activity to the mammalian PLC-δ isofrom since it preferentially hydrolyzes PIP2 over PI in a Ca2+-dependent manner, to produce IP3 and DAG. CnPlc1 activity is consistent with the activity and substrate preference of other eukaryotic PLC enzymes and the fact that hallmark domains of eukaryotic PLC proteins, the X and Y catalytic domains, and a C2 calcium binding motif are present in CnPlc1 (32). In mammalian cells, the C2 motif is involved in calcium-dependent binding of PLC to membrane phospholipids and orientation of the catalytic domain toward the membrane surface (33). The ability of CnPlc1 to bind to the plasma membrane via its C2 domain would enable Plc1 to sense and respond to cell surface perturbations arising due to changes in the external environment. *C. neoformans* also has a second PLC, Plc2, which has a catalytic domain similar to bacterial PLCs, suggesting the functional similarity to prokaryotic enzymes. However, deletion of Plc2 produced no changes in cellular function or virulence (4).

We identified two putative inositol polyphosphate multikinasases in the *C. neoformans* genome, designated Arg1 and Arg2, with Arg1 being the closest homolog of ScArg82. Accumulation of IP3 in the Δarg1 mutant, but not in the Δarg2 mutant, established Arg1 as the major IP3 kinase in *C. neoformans*. This observation coupled with the fact that IP3 content is reduced in Δplc1 cells, confirms that there is a block within the IP-processing pathway in both mutants. These data also demonstrate that Plc1 and Arg1 function within the same signaling pathway, with Plc1 providing a substrate (IP3) for Arg1.

While accumulation of the Plc1 substrate IP3 was expected in Δplc1 cells, even higher IP3 levels were detected in Δarg1 cells. A similar phenomenon was observed in the ScΔarg82 mutant (23). It is possible that the excessive IP3 in CnΔarg1 competes with IP2 for binding to the C2 domain of Plc1 and thus inhibits Plc1 enzyme activity. This hypothesis is supported by the finding that the C2 domain-containing PLC of a squid binds IP3 (34). A functionally similar phenomenon of feedback inhibition was described for mammalian PLC-δ1, which binds IP3-containing membranes via its pleckstrin homology domain. The IP3 produced following receptor-stimulated activation of PLC-δ1 antagonizes its binding to PI(4,5)P2, causing translocation of PLC-δ1 from the plasma membrane to the cytosol and rendering it inactive (35, 36).

Common phenotypic defects of the Δarg1 and Δplc1 mutants, including sterility, compromised cell wall, thermostability, reduced capsule size, and melanization, may be caused by accumulation of PI(4,5)P2 or absence of complex IPs. In *S. cerevisiae*, the lack of detectable PP-IPs in Δarg82 and Δkcs1 mutants is thought to be responsible for vacuolar fragmentation, salt stress sensitivity, and a defective cell wall (22). However, excessive accumulation of PI(4,5)P2 is also detrimental, as deletion of PI(4,5)P2-phosphorylating synaptojamins in *S. cerevisiae* caused defects in actin organization, endocytosis, and clathrin-mediated sorting between the Golgi apparatus and endosomes (21). It should be noted that despite the overall phenotypic similarity between the *C. neoformans* Δplc1 and Δarg1 mutants, the extent of each defect was not the same. The Δarg1 mutant was more sensitive than the Δplc1 mutant to a growth temperature of 37°C and did not produce any detectable melanin, while the Δplc1 mutant was slightly pigmented. The virulence of the Δplc1 mutant and the propagation in moth larvae were markedly reduced, while the Δarg1 mutant propagated and caused insect death, albeit more slowly than the WT. These differences may be attributed to the opposing trend in IP3 content in the two mutants and/or the more significant accumulation of PI(4,5)P2 in the Δarg1 mutant.

Consistent with defects in pathogenicity-related phenotypes, virulence of both Δplc1 and Δarg1 strains was attenuated in *G. mellonella*, which is a proven invertebrate model of cryptococcosis (37). Larvae infected with the Δplc1 mutant survived longer than larvae infected with the Δarg1 mutant, despite the Δplc1 strain having a higher growth rate than the Δarg1 strain at 30°C in vitro on a standard growth medium. This difference in survival correlated with the extent of propagation of each strain within the host: while both the WT and Δarg1 strains underwent significant propagation, the Δplc1 strain did not. Differences in the ability of the Δplc1 and Δarg1 strains to assimilate nutrients and/or maintain viability within the host may be responsible for the difference in the virulence between these mutants, as the nutritional environment in larval hemocoel differs significantly from that of standard liquid media used to propagate yeast in vitro. In support of this hypothesis, the ScΔplc1 strain exhibits compromised ability to assimilate galactose, raffinose, and glycerol (5) and rapidly loses viability in the absence of a nitrogen source (5).

As Plc1-mediated hydrolysis of PIP2 also generates DAG, it is possible that some of the phenotypic abnormalities of the Δplc1 mutant are caused by the reduction in DAG content. Moreover, if Plc1 is inhibited in the Δarg1 mutant by the excess of IP3, the same defect might be expected in both mutants. However, we propose that it is unlikely that a reduction in DAG content in both mutants is responsible for their phenotypic similarity: first, as the *S. cerevisiae* Δplc1, Δarg1, and Δkcs1 strains share a significant degree of similarity, the absence of PP-IPs is more likely to be the major cause of common phenotypic defects in these mutants (38, 39). Second, PLCs is not the only source of DAG in *C. neoformans*: DAG is also produced by Ipc1 (inositol-phosphorylceramide synthase-1), an enzyme of the sphingolipid biosynthetic pathway. Ipc1-derived DAG was shown to facilitate *C. neoformans* protein kinase C activation and subsequent melanization (40, 41).

In *S. cerevisiae*, the absence of Plc1, Arg82, and Kcs1 caused an
increase in cell size and vacuolar fragmentation. Although the cells of the *C. neoformans* Δarg1 mutant were also enlarged, we observed the opposite defect in vacuolar morphology: one large vacuole dominated the mutant cells, in contrast to multiple small vacuoles observed in the WT. This defect was significantly more pronounced in the Δarg1 cells than in the Δppl1 cells. As PIP2 plays an essential role in vacuole fusion in *S. cerevisiae* (42), elevated PIP2 in the *C. neoformans* Δarg1 strain and, to a lesser extent, in the Δppl1 strain may be responsible for the enlarged vacuoles in these mutants.

We observed a dramatic cell separation defect in both Δppl1 and Δarg1 cells. Postmitotic mother and daughter cells remained fused, often forming new buds. Interestingly, a similar defect was observed in an *S. pombe* Δipk1 mutant, but not in *S. cerevisiae* (25). *S. pombe* Δipk1 cells were defective in dissolution of the septum and the cell wall surrounding the septum, resulting in accumulation of binuclear cells with a medial division septum. This defect was attributed to the absence of *IP6* and *IP7* in the *S. pombe* Δipk1 strain, a feature likely to be shared by the *C. neoformans* Δppl1 and Δarg1 mutants. As phosphoinositides and IPs are involved in multiple aspects of intracellular trafficking, we tracked the endocytic pathway in *C. neoformans* using the lipophilic styryl dye FM4-64. Endocytosis is initiated at the plasma membrane, which invaginates to form an endocytic vesicle. The endocytic vesicle is then transferred to an endosomal compartment that subsequently fuses with vacuoles. However, in the *S. cerevisiae* Δarg1 and Δkcs1 mutants, FM4-64 accumulated in aberrant endosomal intermediates juxtaposed to vacuoles (23). In contrast, the *C. neoformans* Δarg1 mutant accumulated FM4-64 in large vacuoles, which were typical for this mutant, but rarely appeared in wild-type cells. Interestingly, the internalization of FM4-64 was considerably slower in Δppl1 cells than in wild-type and Δarg1 cells. The striking differences in vacuolar morphology and endocytosis between the *S. cerevisiae* Δarg2 and *C. neoformans* Δarg1 mutants, despite the similarity of their IP3/PIP2 profiles, imply differences in the regulation of vacuole fusion and/or intracellular trafficking in pathogenic basidiomycetes such as *C. neoformans* and the nonpathogenic ascomycete *S. cerevisiae*.

In mammalian cells, Ptc1-derived IP3 serves as a precursor for complex IPs, but it also triggers calcium release and subsequent calcineurin activation. Yeast, however, does not possess orthologs of mammalian IP3 receptors, and the ability of PLC to activate calcineurin in yeast is unclear. The limited phenotypic similarity between the CnΔppl1 mutant and the calcineurin (Δcna1) mutant clearly indicates that calcineurin does not depend solely on Ptc1 for its activation. We therefore tested the possibility that Ptc1 partially contributes to calcineurin activation. Inhibition of calcineurin in the Δppl1 strain at 25°C caused growth retardation, indicating that under conditions that do not normally require calcineurin function, calcineurin is essential to compensate for the absence of Ptc1. We previously demonstrated that the chin intron trans-encoding gene *CHS6* is regulated by calcineurin and highly expressed in the presence of calciofluor white (12). Although expression of *CHS6* was lower in the Δppl1 strain than in the wild type, inhibition of calcineurin in the Δppl1 strain caused a dramatic reduction in *CHS6* expression, indicating that calcineurin is active in the Δppl1 strain. To test whether some of the Δppl1 abnormalities, which are shared by the Δcna1 mutant, are due to reduced calcineurin activation, we attempted to rescue these defects by providing extracellular calcium, which activates calcineurin in *C. neoformans* (12). However, none of the Δppl1 defects tested was rectified by extracellular calcium. Taken together, these findings suggest that Ptc1 does not contribute significantly to calcineurin activity. Similar to Ptc1 and Cna1, Arg1 is essential for growth at 37°C, formation of mating filaments, and cell wall integrity. It is therefore likely that the Ptc1/IPK pathway functions in parallel to calcineurin to regulate these essential functions, although there may potentially be cross talk between the two pathways. This redundancy most likely contributes to the robustness of *C. neoformans* and its success as a pathogen.

In summary, our study shows that *C. neoformans* Ptc1 produces IP3, which serves as a precursor for the synthesis of more complex IPs by the inositol polyphosphate multikinase Arg1. Arg1-generated IPs are likely to be further phosphorylated, as the *C. neoformans* genome encodes inositol hexa- and heptakisphosphate kinases. Ptc1, Arg1, and their products play multiple roles in cellular homeostasis and have a dramatic impact on virulence of *C. neoformans*. The role of IPs and their products has never been addressed in pathogenic fungi, and our findings lay the foundation for investigation of the mechanisms by which IPs and IP metabolism promote fungal virulence.

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