The fungus *Cryptococcus neoformans* is an opportunistic human pathogen that causes a life-threatening meningoencephalitis by expression of virulence factors such as melanin, a black pigment produced by the cell wall-associated enzyme laccase. In previous studies (Heung, L. J., Luberto, C., Plowden, A., Hannun, Y. A., and Del Poeta, M. (2004) *J. Biol. Chem.* 279, 21144–21153) we proposed that the sphingolipid enzyme inositol-phosphoryl ceramide synthase 1 (*Ipc1*) regulates melanin production through the generation of diacylglycerol (DAG), which was found to activate *in vitro* protein kinase C1 (*Pkc1*). Here, we investigated the molecular mechanisms by which DAG regulates *Pkc1* activity and the effect of this regulation on laccase activity and melanin synthesis. To this end we deleted the putative DAG binding C1 domain of *C. neoformans* *Pkc1* and found that the C1 deletion abolished the activation of *Pkc1* by DAG. Deletion of the C1 domain repressed laccase activity and, consequently, melanin production. Finally, we show that these biological effects observed in the C1 deletion mutant are mediated by alteration of cell wall integrity and displacement of laccase from the cell wall. These studies define novel molecular mechanisms addressing *Pkc1*-laccase regulation by the sphingolipid pathway of *C. neoformans*, with important implications for understanding and targeting the *Ipc1*-Pkc1-laccase cascade as a regulator of virulence of this important human pathogen.

*Cryptococcus neoformans* is a fungal pathogen that infects humans via the respiratory tract. Dissemination of the infection leads to development of a life-threatening meningoencephalitis, particularly in immunocompromised patients. An important factor that enables *C. neoformans* to cause disease is the pigment melanin, which is proposed to protect the fungus from the host immune response (1–3) and function as an immunomodulator (4). Laccase, a cell wall-associated enzyme encoded by the *CNLAC1* gene (5, 6), catalyzes the formation of melanin from exogenous diphenolic substrates (7). A recent study by Noverr et al. (8) has demonstrated that laccase is required for the dissemination of *C. neoformans* from the lung to the brain, thus suggesting a role for melanin not only in the establishment of infection but also in the progression of disease.

Despite the importance of laccase and melanin to the pathogenicity of *C. neoformans*, relatively little is known about the molecular mechanisms by which they are regulated. A few signaling molecules, including the mitogen-activated protein kinase signaling cascade component STE12 (9, 10) and cyclodiphein A (11), have proposed roles in the regulation of laccase. The Gα protein-cAMP-protein kinase A pathway has also been shown to regulate melanin production by *C. neoformans* (12–14). More recently, we have identified a novel regulatory pathway initiated by the sphingolipid enzyme inositol-phosphoryl ceramide synthase (*Ipc1*),1 which transfers an inositol-phosphoryl moiety from phytadiynolinsitol to phytyceramide to produce inositol-phosphorylceramide (IPC) and diacylglycerol (DAG). We found that the effects of *Ipc1* on melanin production are potentially propagated through the generation of DAG and the subsequent activation of protein kinase C1 (*Pkc1*) (15).

The sphingolipid pathway is an important source of cell signaling molecules not only in mammalian cells but also in fungal cells. For example, the sphingolipid pathway has crucial roles in the heat stress response (16, 17) and endocytosis (18, 19) in the yeast *Saccharomyces cerevisiae*. The roles and mechanisms of sphingolipid-metabolizing enzymes in pathogenic fungi, however, have not been extensively explored. The putative *Ipc1*-DAG-Pkc1 pathway is the first evidence that the sphingolipid pathway is crucial to the regulation of fungal virulence. Additionally, the existence of this pathway establishes a key role for DAG generated from sphingolipid metabolism in eukaryotic signal transduction.

*C. neoformans* *Pkc1* contains a putative DAG binding domain, or C1 domain, which is highly homologous to the C1 domain of DAG-dependent mammalian PKCs (15). The role of the C1 domain as a mediator of DAG-Pkc1 signaling in fungal organisms is not well defined. Whereas biochemical data suggests that *C. neoformans* *Pkc1* is activated by DAG (15), fungal *Pkc1* homologs from *S. cerevisiae* and *Candida albicans* are not regulated by DAG (20–22). Mammalian PKC isoforms regulated by DAG have conserved residues in the C1 domain required for DAG activation (Pro11, Gly20, and Gln27) as well as five hydrophobic residues surrounding the DAG-binding site (positions 8, 13, 20, 22, and 24) (23). The three DAG-binding site residues and four of the five hydrophobic residues are

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1 The abbreviations used are: *Ipc1*, inositol-phosphorylceramide synthase 1; IPC, inositol-phosphorylceramide; DAG, diacylglycerol; *Pkc1*, protein kinase C1; NATI, nourseothricin acetyltransferase 1; MOPS, 4-morpholinepropanesulfonic acid; DPG, dipalmitoylglycerol; WT, wild type.
conserved in the C1 domain of C. neoformans Pkc1 (15) but largely absent from the C1 domains of S. cerevisiae and C. albicans Pkc1 enzymes (24). Thus, C. neoformans Pkc1 presents a unique opportunity to study the role of the C1 domain as a site of DAG regulation in a fungal system.

In the present study we investigated the *in vivo* mechanism of DAG-Pkc1 signaling and the role of this regulation in laccase activity and melanin formation. We found that the C1 domain is required for the activation of Pkc1, and activity and melanin formation. We found that the C1 domain of DAG-Pkc1 signaling and the role of this regulation in laccase 

**Lipid Extraction**—Approximately 5 x 10^6 cells were collected from overnight liquid cultures, and lipids were extracted by the method of Bligh and Dyer (26). The amount (mmol) of inorganic phosphate (P) in each sample was measured as previously described (27). Triton-X-100/ lipid mixed micelles were prepared (28) with 8 mol% (based on F, measurements) and used in the *in vitro* kinase assay with recombinant C. neoformans Pkc1 protein, which was expressed in S. cerevisiae and immunoprecipitated as described previously (15).

**Tagging of PCK1 Gene**—To facilitate the study of endogenously expressed C. neoformans, the PKC1 gene was tagged with the V5 epitope (YP) or YNB media supplemented with 2% galactose or 2% glucose was used for the up- and down-regulation of IPC1 expression in the GAL7::IPC1 strain. All chemical reagents were obtained from Sigma unless otherwise noted.

**Strains, Growth Media, and Reagents—** C. neoformans var. grubii serotype A strain H99 (wild type) and C. neoformans strain GAL7::IPC1 (25) were routinely grown in yeast extract/peptone/dextrose medium or Difco basal medium (YNB, 2% glucose). Yeast extract/peptone (YP) or YNB media supplemented with 2% galactose or 2% glucose was used for the up- and down-regulation of IPC1 expression in the GAL7::IPC1 strain. All chemical reagents were obtained from Sigma unless otherwise noted.

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and stained with Calcofluor™ White M2R (Molecular Probes, Eugene, OR). Cells were observed by UV light microscopy.

For transmission electron microscopy cells were pelleted and fixed in 2% cacodylate glutaraldehyde for 1 h, rinsed overnight in cacodylate buffer, post-fixed in 2% aqueous osmium tetroxide for 1 h, and rinsed in distilled water. Samples were then dehydrated in ascending concentrations of ethanol: 50% EtOH for 5 min, 70% EtOH for 15 min, 85% EtOH for 15 min each. Transition to infiltration was begun using the intermediate fluid, propylene oxide, for 2 changes of 15 min each. Samples were first infiltrated with a 1:1 solution of propylene oxide and Embed 812 embedding resin for 1 h and continued with a 1:2 solution of propylene oxide and Embed 812 overnight. Samples were embedded in pure Embed 812 and cured in a 60 °C oven for 48 h. Thin sections of 70 nm were cut using a Reichert ultramicrotome and collected on copper grids. The heavy metal salts of uranyl acetate and lead citrate were used for staining, and the sections were examined using a JEOL JEM1210 electron microscope. Supplies for electron microscopy were obtained from Electron Microscopy Sciences (Fort Washington, PA).

Cell Wall Extraction—Crude cell wall preparations were made similar to the method used by Zhu et al. (5) with minor modifications. Briefly, cells were collected and resuspended in lysis buffer (50 mM Tris-Cl, pH 7.5 and 1 mM phenylmethylsulfonyl fluoride). Acid-washed glass beads (Sigma) were added in a volume equal to 3/4 of the cell volume, and the cells were homogenized in a Mini-Beadbeater (Bio-Spec Products, Bartlesville, OK) 3 times for 45 s at 4 °C. Total cell lysate was aspirated from the beads and transferred to a new tube. Beads were washed once with lysis buffer, and the wash liquid was added to the tube containing the cell lysate. Microscopic observation confirmed complete lysis of cells. The lysate was then centrifuged at 16,000 × g for 30 min at 4 °C. The pellet was designated the cell wall fraction and separated from the supernatant. The cell wall fraction was resuspended in 10 mM sodium phosphate, pH 7.0, with 2% SDS and boiled for 10 min. After trichloroacetic acid precipitation of both cell wall and supernatant fractions, protein pellets were resuspended in Laemmli buffer by boiling for 10 min.

Capsule Induction—C. neoformans strains IPC1/PKC1::V5 and IPC1/C1-PKC1::V5 were grown in YNB media supplemented with 2% glucose for 24 h in a 30 °C shaker. Cells were pelleted, resuspended, and inoculated into induction media (3 gliter BBL™ Sabouraud liquid broth modified antibiotic medium 13 (BD Biosciences), 50 mM MOPS, pH 7.3 at 2.5 × 10^6 cells/ml. Cultures were grown for 40 h in a 30 °C shaker, stained with India ink, and observed by light microscopy. To measure capsule size, pictures were taken of five different fields of each sample at 40× magnification, and the areas of the capsules of 20 cells from each field (100 total cells) were measured using LabWorks software (UVP, Inc., Upland, CA).

Western Blot Analysis—Protein samples were loaded on SDS-polyacrylamide gels, separated by electrophoresis, and transferred onto Immuno-Blot™ polyvinylidine difluoride membranes (Bio-Rad). Pkc1 and ΔC1-PKC1 proteins tagged with the V5 epitope were detected using anti-V5 antibody at 1:2000 dilution in 5% milk, 1× phosphate-buffered saline and 0.1% Tween 20. Laccase was detected using anti-laccase monoclonal antibody clone G3P4D3 (a gift from Dr. Peter Williamson, University of Illinois at Chicago College of Medicine) at 2.6 μg/ml in 3% bovine serum albumin, 1× phosphate-buffered saline and 0.1% Tween 20.

**RESULTS**

**Lipids Modulated by Ipcl Regulate Activation of C. neoformans Pkc1**—Because our previous studies indicated that Ipcl modulates DAG and phytoceramide levels in C. neoformans cells and that Pkc1 activity is regulated by DAG and phytoceramide in vitro (15), we next investigated whether modulation of Ipcl in C. neoformans cells regulates the activity of Pkc1. Therefore, Ipcl expression was modulated in the GAL7::IPC1 strain by growing cells in the presence of glucose (repressing conditions) or galactose (inducing conditions). As a control the wild type strain H99 was grown under the same conditions. Next, total lipids were extracted from each of the cultures, and their effect on the activity of recombinant C. neoformans Pkc1 was measured by in vitro kinase assay (Fig. 1). When Pkc1 was treated with lipids from cells in which Ipcl was down-regulated (GAL7::IPC1 grown on glucose), kinase activity decreased by 30.7% relative to treatment with lipids from the wild type cells grown in glucose (p < 0.05). Conversely, when Pkc1 was treated with lipids from cells in which Ipcl was up-regulated (GAL7::IPC1 grown on galactose), kinase activity increased by 21.4% compared with treatment with lipids from wild type cells grown on galactose. No difference in Pkc1 activity was observed between treatment with lipids from the wild type cells grown in glucose or galactose. Therefore, the lipids that are modulated by Ipcl in vivo regulate the activity of C. neoformans Pkc1.

**Deletion of C1 Domain from Pkc1 in C. neoformans**—To study whether the putative DAG binding domain, or C1 domain, plays a role in the regulation of Pkc1 in C. neoformans, the C1 domain was deleted from the PKC1 locus, and a V5 epitope tag was fused to the C terminus (Fig. 2A) in both the wild type and GAL7::IPC1 strains. We detected a single band at 120 kDa in the wild type and GAL7::IPC1 strains, yielding Cryptococcus strains IPC1/PKC1::V5 and GAL7::IPC1/C1-PKC1::V5 (Fig. 2B). The wild type Pkc1 gene was also tagged with the V5 epitope (Fig. 2C) in the wild type and GAL7::IPC1 strains, yielding Cryptococcus strains IPC1/PKC1::V5 and GAL7::IPC1/PKC1::V5 (Fig. 2D). As illustrated in Fig. 2E, we detected a single band at ~120 kDa, the predicted size of Pkc1 including the V5 epitope tag, in the IPC1/PKC1::V5 and GAL7::IPC1/PKC1::V5 strains. In the IPC1/C1-PKC1::V5 and GAL7::IPC1/C1-PKC1::V5 strains, a single band at ~110 kDa is detected, which is the expected size of the ΔC1-PKC1 protein fused to the V5 epitope. The expression of the ΔC1-PKC1::V5 protein was similar to that of the PKC1::V5 protein in the background of both parental strains. We next examined whether deletion of the C1 domain would affect the
growth of *Cryptococcus* and found that there were no significant changes in growth between the wild type, GAL7::IPC1, IPC1::PKC1::V5, and GAL7::IPC1::PKC1::V5 strains in YP-galactose plates at 30 °C (data not shown).

**Deletion of the C1 Domain Significantly Represses Laccase Activity and Melanin Production in C. neoformans**—Because the IPC1::V5::PKC1 pathway regulates melanin production by *C. neoformans* (15) and the data above suggest that the C1 domain is required for the activation of Pkc1 by DAG, we investigated the effect of deletion of the C1 domain on melanin formation. First, the laccase activities of the parental strains H99 (WT) and GAL7::IPC1 and of the ΔC1 mutant strains IPC1::ΔC1-PKC1 and GAL7::IPC1::ΔC1-PKC1 were measured (Fig. 4A). Laccase activity of IPC1::ΔC1-PKC1 was reduced by ~65% compared with the parental strain H99 (WT) (p < 0.03). Similarly, laccase activity of the GAL7::IPC1::ΔC1-PKC1 strain was lower by ~34% than the parental strain GAL7::IPC1 (p < 0.03). Under these inducing (galactose) conditions, the laccase activity of GAL7::IPC1 was higher than that of wild type H99 due to the up-regulation of Ipc1, as expected (p < 0.01).

To confirm this biochemical regulation at the biological level, melanin production was measured by growing cells on galactose plates containing L-3,4-dihydroxyphenylalanine, a substrate for the laccase enzyme. As illustrated in Fig. 4B, the strain IPC1::ΔC1-PKC1 produced less melanin pigment compared with the parental wild type strain H99 (WT), and GAL7::IPC1::ΔC1-PKC1 produced less melanin than parental strain GAL7::IPC1. Under these conditions, the GAL7::IPC1 strain, in which IPC1 is up-regulated, produced more melanin than the wild type, in which IPC1 is not modulated, as expected. The decreases in both laccase activity and melanin production were confirmed using a second independent
DAG-Pkc1 Signaling in Fungal Melanogenesis

Fig. 3. Effect of deletion of the C1 domain on activation of Pkc1 by DAG. Deletion of C1 domain abolishes activation of Pkc1 by DAG. Shown are an autoradiogram and the results from PhosphorImager quantitation of histone phosphorylated by C. neofor m ans Pkc1 and ΔC1-PKC1 proteins. DAG induced a 2-fold increase in Pkc1 activity. The specific activity of Pkc1 in the absence of lipids (−) was 14.2 ± 4.1 pmol of histone phosphorylated/min/mg of lysate protein. ΔC1-PKC1 was not activated by DAG. The specific activity of ΔC1-PKC1 in the absence of lipids was 17.1 ± 3.7 pmol of histone phosphorylated/min/mg of lysate protein. Data are the geometric means ± S.D. of three independent experiments. Autoradiograms are representative of three separate experiments. *, p < 0.05.

Fig. 4. Consequence of C1 deletion on laccase activity and melanin production by C. neofor m ans. A, Under galactose conditions the ΔC1 mutant strains have decreased laccase activity compared with their parental WT and GAL7::IPC1 strains. Data are the geometric means ± S.D. of three separate experiments. *, p < 0.03, IPC1/ΔC1-PKC1 strain versus IPC1/PKC1 strain; †, p < 0.03, GAL7::IPC1/ΔC1-PKC1 strain versus GAL7::IPC1/PKC1 strain. ‡, p < 0.01, GAL7::IPC1/PKC1 versus IPC1/PKC1. B, Growth of these strains on 1,3,4-dihydroxyphenylalanine (l-DOPA)/galactose agar plates shows decreased melanization by the ΔC1 strains compared with the parental strains. Pictures are representative of three independent experiments.

GAL7::IPC1/ΔC1-PKC1 transformant (data not shown).

ΔC1 Mutants Exhibit Defects in Cell Wall Integrity—Because laccase is a cell wall-associated enzyme (5) and in S. cerevisiae Pkc1-mediated pathways regulate cell wall integrity (32), we investigated whether the Ipc1-Pkc1 pathway in C. neofor m ans may regulate laccase activity by retaining the enzyme in the cell wall. To determine whether the Ipc1-Pkc1 pathway plays a role in cell wall integrity, the wild type, GAL7::IPC1, and their ΔC1 mutant strains were tested for sensitivity to lysing enzymes, which digest components of the cell wall. After treatment with lysing enzymes, the number of spheroplasts, or cells lacking the cell wall, was counted. Down-regulation of Ipc1 in the GAL7::IPC1 strain increased the number of spheroplasts by 76.2% (p < 0.01), whereas up-regulation of Ipc1 caused a 62.8% decrease in the number of spheroplasts compared with the wild type strain (p < 0.01). There was no difference in the number of spheroplasts between the wild type strains grown in glucose versus galactose media (Fig. 5A). When the C1 domain was deleted from Pkc1 in the wild type strain, the number of spheroplasts increased by 64.9% (p < 0.01). When the C1 domain was deleted in the GAL7::IPC1 strain, the decrease in spheroplasts caused by the up-regulation of Ipc1 was abolished, and the number of spheroplasts returned to the wild type level (Fig. 5B) (p < 0.05). Therefore, modulation of Ipc1 regulates the susceptibility of C. neofor m ans cell wall to digestion, and deletion of the C1 domain increases this susceptibility.

To further test for cell wall defects, the ability of C. neofor m ans strains to grow in the presence of the detergent SDS was measured. Serial dilutions of the ΔC1 mutants and their parental strains were spotted onto YP plates containing 0.05% SDS and 2% of either glucose or galactose (Fig. 6). The results show that up-regulation of Ipc1 in the GAL7::IPC1 strain increased growth in the presence of SDS compared with the wild type strain (compare lane 3 to lane 1 in galactose). Conversely, down-regulation of Ipc1 in the GAL7::IPC1 strain repressed growth compared with the wild type strain (compare lane 3 to lane 1 in galactose). In the wild type strain, cells exhibited reduced growth when the C1 domain was deleted from Pkc1 (compare lanes 2 and 1) in either glucose or galactose media. As expected, deletion of the C1 domain in the GAL7::IPC1 strain reduced growth under the condition of Ipc1 up-regulation (compare lane 4 to lane 3 in galactose). Interestingly, the C1 deletion slightly enhanced growth when Ipc1 is down-regulated (compare lanes 4 to 3 in glucose). On plates lacking SDS, all strains had similar growth in either glucose or galactose (data not shown).

ΔC1 Mutants Display Changes in Cell Wall and Capsule Morphology—To examine directly whether ΔC1 mutants exhibit defects in the cell wall structure, C. neofor m ans wild type and IPC1/ΔC1-PKC1 cells were stained with calcofluor white, which detects chitin, a component of fungal cell walls (33), and cells were observed under UV light microscope (Fig. 7). By counting ~270 cells in at least four different fields, we found that 21.4 ± 0.9% of budding cells of the wild type strain exhib-
GAL7/IPC1 determinations. *, compared with WT. Data are the geometric means grown in galactose) decreases the number of spheroplasts by 62.8% compared with the WT strain. Up-regulation of Ipc1 (GAL7::IPC1 strain grown in glucose) increases the number of spheroplasts by 76.2% treated with lysing enzymes. Down-regulation of Ipc1 (glucose, Ipc1 modulation and deletion of the C1 domain.

Data are representative of three independent experiments.

FIG. 5. Sensitivity of C. neoformans to cell wall digestion upon modulation of Ipc1 and deletion of C1 domain. A, modulation of Ipc1 regulates spheroplast formation when C. neoformans cells are treated with lysing enzymes. Down-regulation of Ipc1 (GAL7::IPC1 strain grown in glucose) increases the number of spheroplasts by 76.2% compared with the WT strain. Up-regulation of Ipc1 (GAL7::IPC1 strain grown in galactose) decreases the number of spheroplasts by 62.8% compared with WT. Data are the geometric means ± S.D. of five determinations. *, p < 0.01, GAL7::IPC1 versus WT in glucose. †, p < 0.01, GAL7::IPC1 versus WT in galactose. B, deletion of the C1 domain from both the WT and GAL7::IPC1 strains renders cells more sensitive to lysing enzymes, resulting in increased spheroplast formation. Data are the geometric means ± S.D. of four determinations. *, p < 0.01, IPC1/ΔC1-PKC1 strain versus IPC1/PKC1 strain. †, p < 0.05, GAL7::IPC1/ΔC1-PKC1 strain versus GAL7::IPC1/PKC1 strain.

FIG. 6. Growth of C. neoformans in the presence of SDS upon Ipc1 modulation and deletion of the C1 domain. Cells were spotted and grown on agar plates containing 0.05% SDS and either glucose or galactose at 30 °C. Down-regulation of Ipc1 in GAL7::IPC1 (glucose, row 3) decreases growth compared with the WT (glucose, row 1), whereas up-regulation of Ipc1 in galactose, row 3) slightly increases growth compared with WT (galactose, row 1). When Ipc1 is not modulated, deletion of the C1 domain from Pkc1 (row 2) decreases growth compared with when Pkc1 is intact (row 1) in both glucose and galactose. When Ipc1 is up-regulated, deletion of the C1 domain (galactose, row 4) decreases growth compared with when Pkc1 is intact (galactose, row 3). When Ipc1 is down-regulated, deletion of the C1 domain (glucose, row 4) increases growth compared with when Pkc1 is intact (glucose, row 3). Data are representative of three independent experiments.

FIG. 7. Chitin distribution within cell wall of C. neoformans wild type and ΔC1 strains. Cells of the WT IPC1/PKC1 and IPC1/ΔC1-PKC1 strains were stained with calcofluor white, a stain for fungal chitin, and observed under UV light. 21.4 ± 9.9% of budding cells of the WT strain show intense staining within the bud neck (arrowheads), whereas only 15.9 ± 0.67% of ΔC1 budding cells exhibit this staining. Because the capsule of C. neoformans is anchored to cell wall components (34), we investigated whether the ΔC1 mutants exhibit any changes in capsule formation. Using India ink staining, we observed that overall capsule size was decreased by ~42% in the IPC1/ΔC1-PKC1 mutant strain compared with the parental IPC1/PKC1 strain (data not shown).

To further investigate whether structural alteration of the capsule was associated with the absence of the C1 domain, the wild type, GAL7::IPC1, IPC1/ΔC1-PKC1, and GAL7::IPC1/ΔC1-PKC1 strains were examined by transmission electron microscopy. As shown in Fig. 8, the ΔC1 mutants exhibited reduced density and length of capsule microfibrils radiating from the cell wall compared with the parental strains. No significant differences in cell wall size were observed between the ΔC1 mutants and their parental strains by transmission electron microscopy. All together, these results suggest that signal transduction through the C1 domain regulates capsule formation and may modulate components of the cell wall.

Deletion of the C1 Domain Disrupts Localization of Laccase to the Cell Wall—C. neoformans cells were lysed, and a crude cell wall fraction was separated from the remaining supernatant. The localization of laccase to each fraction was detected by Western blot using antibodies directed against the laccase enzyme (Fig. 9). In the wild type strain, the majority of laccase was associated with the cell wall. However, when the C1 domain was deleted, laccase was primarily localized in the supernatant. The total level of laccase protein did not change significantly between the wild type and IPC1/ΔC1-PKC1 strains.
In vivo DAG-Pkc1 signaling was further investigated by selective deletion of the C1 domain from the *C. neoformans* PKC1 locus to express endogenous Pkc1 protein that lacked only the residues comprising the C1 domain (Fig. 2). Deletion of the C1 domain abolished the activation of Pkc1 by DAG (Fig. 3) and resulted in significant decreases of laccase activity and production of melanin pigment (Fig. 4), indicating that transduction through the Ipc1-DAG-Pkc1 cascade regulates melanogenesis in *in vivo*.

The mechanism by which the Ipc1-DAG-Pkc1 pathway regulates the laccase enzyme was then examined. Because laccase is a cell wall-associated enzyme (5) and Pkc1 regulates cell wall integrity in yeasts (32), we hypothesized that the Ipc1-DAG-Pkc1 pathway may control integrity of the cell wall. We first investigated whether the Ipc1-DAG-Pkc1 pathway plays a role in the regulation of cell wall integrity in *C. neoformans*. We found that the sensitivity of *C. neoformans* to cell wall-digesting enzymes is modulated by Ipc1 (Fig. 5A), and importantly, deletion of the C1 domain increased this susceptibility to digestion (Fig. 5B). Another phenotype of cells with wall defects is decreased growth in the presence of SDS. We found that modulation of Ipc1 regulates growth in the presence of SDS, and deletion of the C1 domain increases the sensitivity to SDS (Fig. 6). Additionally, direct observation of cells stained for chitin in the cell wall revealed that deletion of the C1 domain causes a slight decrease in chitin deposition, particularly in the septum dividing the mother cell from the bud (Fig. 7). Although the ΔC1 mutant strains did not exhibit any obvious defects in budding or division, the changes in septal chitin suggest that deletion of the C1 domain causes some perturbation in the composition of the cell wall. Examination of cells by transmission electron microscopy did not reveal any significant changes in cell wall size. However, this observation again does not preclude any ultrastructural changes in the cell wall. Previously, it was observed in *S. cerevisiae* that down-regulation of Ipc1 causes defects in cell wall deposition (35) and that Pkc1 may regulate chitin synthase III (Chs3p) (36). Therefore, the Ipc1-DAG-Pkc1 pathway may control integrity of the cell wall through the synthesis of cell wall components without affecting its overall size.

**FIG. 8.** Transmission electron microscopy of *C. neoformans* parental and ΔC1 strains. Cells were visualized using transmission electron microscopy. The ΔC1 mutants exhibit shorter and sparser capsule microfibrils (inset) compared with the parental strains. The bar represents 500 nm.

**FIG. 9.** Localization of laccase in *C. neoformans* wild type and ΔC1 strains. Western blot analysis of total, supernatant (sup), and cell wall fractions of WT and IPC1/ΔC1-PKC1 strains with anti-laccase monoclonal antibody G3P4D3. Laccase is primarily localized to the cell wall in the WT but is displaced into the supernatant when the C1 domain is deleted. The immunoblot is representative of three independent experiments.

Samples were normalized to total cellular protein measured by Bio-Rad protein assay before fractionation. Equal loading of cellular proteins was confirmed by Ponceau staining of the immunoblot membrane (data not shown).

**DISCUSSION**

In the present study the role and mechanism of the C1 domain, a putative DAG binding domain of Pkc1, in mediating the regulation of melanin production of *C. neoformans* by the sphingolipid enzyme Ipc1 and its product DAG were investigated. The results indicate that deletion of the C1 domain disrupts the regulation of Pkc1 by Ipc1 and DAG and causes a significant decrease in laccase activity and, consequently, melanogenesis. Furthermore, we establish a role for the Ipc1-DAG-Pkc1 pathway in cell wall maintenance and provide evidence that signal transduction through this pathway is required for proper localization of laccase to the cell wall. All together, the data suggest that the C1 domain is the platform through which Ipc1, via DAG, regulates Pkc1 and controls melanin production in *C. neoformans*. Previous data suggested that regulation of Pkc1 is under the control of Ipc1, since levels of DAG and phytoceramide are modulated by Ipc1 and since in vitro biochemical studies show that *C. neoformans* Pkc1 is activated by DAG (a product of Ipc1) and also inhibited by phytoceramide (a substrate for Ipc1) (15). The studies presented here confirm that signal transduction through an Ipc1-DAG-Pkc1 pathway does occur in vivo. When Ipc1 is down- and up-regulated in *C. neoformans* cells, the lipids modulated in vivo inhibit and activate Pkc1, respectively (Fig. 1). The inhibition of Pkc1 upon treatment with lipids extracted from cells in which Ipc1 was down-regulated (GAL7::IPC1 in glucose) is statistically significant, whereas the activation of Pkc1 upon treatment with lipids extracted from cells in which Ipc1 was up-regulated (GAL7::IPC1 in galactose) was not statistically significant although consistently higher than the control in three independent determinations. It must be considered that the assay utilized a total neutral lipids extract, and thus, the lipid molecules affecting Pkc1 activity (phytoceramide and DAG) constitute only a small proportion of the total lipids. Previously, we determined that down-regulation of Ipc1 decreases DAG and increases phytoceramide levels, synergistically leading to a significant inhibition of Pkc1. Interestingly, when Ipc1 is up-regulated, DAG levels are increased, but phytoceramide levels do not change from wild type levels (15). Therefore, under the conditions in which Ipc1 is up-regulated, the activation of Pkc1 is only due to the increase in DAG molecules, the effect of which may be diluted within the total lipid extract. Overall, the results from this experiment support the hypothesis that Ipc1 regulates Pkc1 via lipid metabolites in living cells.
The mechanism by which laccase associates with components of the cell wall is not clear, although studies by Zhu et al. (5) indicate that laccase is covalently attached to the cell wall, potentially through a disulfide or thioester bond. Interestingly, a recent study by Reese and Doering (34) demonstrated that α-1,3-glucan, a component of C. neoformans cell walls, is required for anchoring of the capsule (34). We observed that upon induction of capsule formation, the ΔC1 cells produce a smaller capsule than the wild type strain, as visualized by India ink staining (data not shown). Additional examination of cells using transmission electron microscopy verified that the ΔC1 mutants have decreased length and density of capsule microfibrils compared with the parental strains (Fig. 8). In S. cerevisiae Pkc1 regulates synthesis of cell wall β-glucans (37) (α-glucans are not found in the S. cerevisiae cell wall (38)). Therefore, in C. neoformans Pkc1 may enable proper association of capsular material with the cell wall by regulating production of the cell wall components that anchor the capsule. As a parallel, the Ipc1-DAG-Pkc1 pathway may also regulate the synthesis of cell wall components that serve as an anchor for the laccase enzyme. Further investigation will be required to determine which components of the cell wall are regulated by the Ipc1-DAG-Pkc1 pathway in C. neoformans. However, we do find that disruption of the Ipc1-DAG-Pkc1 pathway prevents localization of laccase to the cell wall (Fig. 9), suggesting that alterations of the cell wall by the Ipc1-DAG-Pkc1 pathway affect the proper anchoring of the laccase enzyme. Because melanin production diminishes when laccase is not associated with the cell wall, our study provides additional information on the biology of this enzyme, indicating that localization of laccase to the cell wall is required for its function and is regulated by the Ipc1-DAG-Pkc1 pathway.

The present work does not exclude other mechanisms by which the Ipc1-DAG-Pkc1 pathway may regulate laccase. Because Pkc1 is a kinase, there is the possibility that laccase is directly bound and phosphorylated by Pkc1. Previous characterization of the laccase enzyme did not reveal that it is a phosphorylated protein (6). Additionally, unpublished data from our laboratory suggest that laccase does not co-immunoprecipitate with Pkc1 and is not phosphorylated in vitro by Pkc1 (data not shown). The investigation into the molecular mechanism by which Pkc1 anchors laccase to the cell wall is the focus of our future studies.

Although the results indicate that the Ipc1-DAG-Pkc1 pathway plays a significant role in the regulation of the cell wall and, consequently, melanin production in C. neoformans, we made several observations that substantiate the existence of additional mechanisms which help modulate these biological effects. Most evident is that deletion of the C1 domain or that a second mechanism is mediating the effects of Ipc1 up-regulation on cell wall and melanogenesis in a C1 domain-independent manner. The first hypothesis is supported by previous studies which found that the inhibitory effect of phytoceramide on Pkc1 was partially diminished after deletion of the C1 domain (15). The second hypothesis is supported by studies in S. cerevisiae which show that genes, such as ECM18, ECM40, and Fig 2 involved in cell wall synthesis and organization, are dysregulated in the absence of sphingolipids (39). Seeing as Ipc1 catalyzes the formation of the complex sphingolipid IPC, it is intriguing to hypothesize that a difference in IPC levels contributes to the phenotypes observed in the two strains.

Other evidence supporting the inhibition of Pkc1 by phytoceramide through the C1 domain is provided by the interesting observation that under Ipc1-repressing (glucose) conditions, the GAL7::IPC1/ΔC1-PKC1 strain exhibited increased, rather than decreased growth in the presence of SDS compared with the parental GAL7::IPC1 strain (see Fig. 6, lanes 3 and 4 in glucose). This result is contrary to what might be expected if the regulation of Pkc1 by Ipc1 was only mediated by DAG through the C1 domain. In previous studies we found that when Ipc1 is down-regulated, both DAG and phytoceramide levels are modulated (DAG is decreased, and phytoceramide is increased) (15). Under these conditions one would predict that Pkc1 would be synergistically inhibited, since phytoceramide also has a negative effect on Pkc1 activity (15) through as yet
unknown molecular mechanisms. However, if phytoceramide exerts any of its inhibitory effects through the C1 domain, deletion of the C1 domain would relieve this inhibition, which could explain why the SDS sensitivity of yeast cells in which Ipcl is down-regulated would be greater when Pkc1 is full-length versus ΔC1-PKC1 (Fig. 6). Partial regulation of Pkc1 by phytoceramide through the C1 domain is supported by studies in mammalian cells which suggest that regulation of PKCs by ceramide may occur through the C1 (40) or the C2 domain (41).

As mentioned above, our earlier in vitro data indicated that inhibition of recombinant C. neoformans Pkc1 by ceramides is slightly attenuated when the C1 domain is deleted (15). This previous result in combination with the data presented here would suggest that phytoceramide exerts some, although not all, of its inhibitory effects through the C1 domain in vivo and, therefore, is an additional mechanism by which Ipcl can regulate the phenotypes attributed to its modulation. Clearly, the biochemical mechanism(s) by which phytoceramide regulates Pkc1 warrants further investigation.

In conclusion, this study demonstrates that the C1 domain of Pkc1 plays an important role in signal transduction initiated by the sphingolipid pathway. The results suggest that activation of Pkc1 by DAG through the C1 domain is a critical signaling event for the proper localization of laccase to the cell wall and that the perturbation of laccase-cell wall association results in alteration of melanin production (a model is presented in Fig. 10). Because melanin is an important virulence factor of C. neoformans, the results of this study have significant implications for understanding the mechanisms by which C. neoformans regulates its pathogenicity, a crucial step toward the development of new anti-fungal therapies.

Acknowledgments—We thank Drs. Yusuf Hannun, Lina Obeid, Edward Balish, and Dhandapani Kuppuswamy for helpful discussions and Dr. Jennifer Schnellmann for editorial assistance. We give special thanks to Carol Moskos and Dr. Debra Hazen-Martin of the Medical University of South Carolina Electron Microscopy Facility for sample preparation and imaging. We also thank Dr. Peter Williamson for helpful comments and the generous gift of the anti-laccase antibody.

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The Role and Mechanism of Diacylglycerol-Protein Kinase C1 Signaling in Melanogenesis by "Cryptococcus neoformans"

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J. Biol. Chem. 2005, 280:28547-28555.
doi: 10.1074/jbc.M503404200 originally published online June 9, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M503404200

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