The Sphingolipid Pathway Regulates Pkc1 through the Formation of Diacylglycerol in Cryptococcus neoformans*

Received for publication, December 1, 2003, and in revised form, February 15, 2004
Published, JBC Papers in Press, March 10, 2004, DOI 10.1074/jbc.M312995200

Lena J. Heung‡, Chiara Luberto‡, Allyson Plowden‡, Yusuf A. Hannun‡, and Maurizio Del Poeta‡§¶
From the Departments of ‡Biochemistry and Molecular Biology and §Microbiology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29425

The sphingolipid biosynthetic pathway generates bioactive molecules crucial to the regulation of mammalian and fungal physiological and pathobiological processes. In previous studies (Luberto, C., Toffaletti, D. L., Wills, E. A., Tucker, S. C., Casadevall, A., Perfect, J. R., Hannun, Y. A., and Del Poeta, M. (2001) Genes Dev. 15, 201–212), we demonstrated that an enzyme of the fungal sphingolipid pathway, Ipc1 (inositol-phosphorylceramide synthase-1), regulates melanin, a pigment required for the pathogenic fungus Cryptococcus neoformans to cause disease. In this study, we investigated the mechanism by which Ipc1 regulates melanin production. Because Ipc1 also catalyzes the production of diacylglycerol (DAG), a physiological activator of the classical and novel isoforms of mammalian protein kinase C (PKC), and because it has been suggested that PKC is required for melanogenesis in mammalian cells, we investigated whether Ipc1 regulates melanin in C. neoformans through the production of DAG and the subsequent activation of Pkc1, the fungal homolog of mammalian PKC. The results show that modulation of Ipc1 regulates the levels of DAG in C. neoformans cells. Next, we demonstrated that C. neoformans Pkc1 is a DAG-activated serine/threonine kinase and that the C1 domain of Pkc1 is necessary for this activation. Finally, through both pharmacological and genetic approaches, we found that inhibition of Pkc1 abolishes melanin formation in C. neoformans. This study identifies a novel signaling pathway in which C. neoformans Ipc1 plays a key role in the activation of Pkc1 through the formation of DAG. Importantly, this pathway is essential for melanin production with implications for the pathogenicity of C. neoformans.

The eukaryotic sphingolipid biosynthetic pathway generates lipids that have important roles in cell signaling in addition to their structural function in cell membranes. Mammalian cer-

amide and sphingosine 1-phosphate regulate many key cellular processes such as the stress response, proliferation, apoptosis, and angiogenesis (1). More recently, fungal sphingolipids have been implicated in the heat stress response (2, 3), endocytosis (4), and signal transduction (6).

Interestingly, both mammalian and fungal sphingolipid pathways also generate diacylglycerol (DAG). 1 DAG is a glycerolipid that is well known for its function as a second messenger and activator of mammalian classical and novel protein kinase C (PKC) isoforms. Sphingolipid enzymes that catalyze the formation of DAG include sphingomyelin synthase in mammalian systems (7–13) and Ipc1 (inositol-phosphorylceramide synthase-1) in fungal systems (14). Sphingomyelin synthase and Ipc1 transfer a phosphate head group from phosphatidylcholine or phosphatidylinositol to ceramide or phytoceramide, respectively. Whether the DAG generated by these sphingolipid-metabolizing enzymes regulates cellular processes is, in fact, not known. In mammalian cells, the activation of sphingomyelin synthase correlates with activation and nuclear translocation of NF-kB (15), events often regulated by DAG-dependent PKC (16). In fungi, the ability of DAG to activate Pkc1, the fungal homolog of mammalian PKC, is still controversial. Ogita et al. (17) and Simon et al. (18) purified a protein kinase from Saccharomyces cerevisiae that was activated by DAG, whereas other studies using the recombinant Pkc1 protein from S. cerevisiae and Candida albicans suggest that Pkc1 is insensitive to DAG (19–21).

Cryptococcus neoformans is a pathogenic fungus that infects mainly immunocompromised patients, and it represents the leading cause of fungal meningoencephalitis worldwide (22). In previous studies, we showed that Ipc1 regulates melanin production through the modulation of laccase (23), the enzyme that, in C. neoformans, oxidizes exogenous diphenolic substrates (e.g. catecholamines) to form melanin (24, 25). Melanin protects the fungus from the host immune response (26), and melanin-deficient mutants of the fungus are indeed avirulent in animal models of cryptococcosis (27). Interestingly, it has been suggested that melanogenesis by mammalian cells is dependent on the activation of PKC by DAG. Initial reports in mammalian melanocytes showed that DAG increases melanin pigmentation in a PKC-dependent manner (28); and more recently, it was demonstrated that PKCβ, an isoform that is activated by DAG, regulates melanin production in mammalian cells (29). Thus, the generation of DAG by Ipc1 raises the intriguing hypothesis that Ipc1 regulates melanin production in C. neoformans through the formation of DAG and the consequent activation of Pkc1.

* This work was supported in part by the Burroughs Wellcome Fund, by Grants AI51924 and AI56168 (to M. D. P.) and Grant HL43707 (to Y. A. H.) from the National Institutes of Health, and by RR17677 Project 2 from the Centers of Biomedical Research Excellence Program of the National Center for Research Resources (to M. D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY137378 and AY137375.

† Burroughs Wellcome New Investigator in Pathogenesis of Infectious Diseases. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., BSB 503, Charleston, SC 29425. Tel.: 843-792-8381; Fax: 843-792-8865; E-mail: delpoeta@musc.edu.

‡ The abbreviations used are: DAG, diacylglycerol; PKC, protein kinase C; l-DOPA, l-3,4-dihydroxyphenylalanine.
Here, we found that modulation of Ipc1 expression regulates the formation of DAG in C. neoformans. Next, we demonstrated that DAG activates C. neoformans Pkc1 and that deletion of a conserved DAG-binding domain abrogates the activation of Pkc1 by DAG. Finally, inhibiting Pkc1 by both pharmacological and genetic approaches inhibits melanin production, thereby establishing a crucial role for Pkc1 in the activation of this virulence factor required for development of infection by C. neoformans.

**EXPERIMENTAL PROCEDURES**

**Strains, Growth Media, and Reagents—**C. neoformans var. grubii serotype A strain H99 (wild type), C. neoformans strain GALT::IPCI, and S. cerevisiae strain JK9-3da (MATα trp1 leu2-3 his3 ura3 ade2 me3) were used in this study. The GAL7::IPCI strain was created from M001, an ade2 isogenic strain derivative of H99, as described previously (25). The C. neoformans H99 and GALT::IPCI strains were routinely grown in yeast extract/potato/dextrose (YPD) medium. Yeast extract/potato (YP) medium supplemented with 2% galactose or with 2% glucose or raffinose was used for up- and down-regulation of IPC1 expression, respectively. S. cerevisiae strain JK9-3da was grown routinely in synthetic medium consisting of 6.7 g/liter yeast nitrogen base (YNB) without amino acids, amino acid mixture lacking uracil (ura-); 0.1 g/liter leucine, 0.4 g/liter serine, 0.2 g/liter threonine, 0.15 g/liter valine, 0.1 g/liter glutamic acid, 0.1 g/liter aspartic acid, 50 mg/liter phenylalanine, 30 mg/liter tyrosine, 30 mg/liter lysine, 30 mg/liter isoleucine, 20 mg/liter histidine, 20 mg/liter arginine, 20 mg/liter methionine, and 0.2 g/liter adenine), 20 g/liter glucose, and 20 g/liter galactose. YNB-ura medium supplemented with 2% galactose or 2% glucose was used to induce or repress transcription by the GALT promoter, respectively. To assess melanin production, L-3,4-dihydroxyphenylalanine (L-DOPA) was used to induce or repress transcription by the GAL1 promoter.

**DAG Kinase Assay—**A total of 10⁸ cells were collected from cultures of the C. neoformans wild-type H99 and GAL7::IPCI strains. Neutral lipids were extracted from the cell pellets as described by Bligh and Dyer (31) and dried down. Three-fourths of each sample were used for lipid measurement, whereas one-fourth was used for inorganic phosphate measurement. Briefly, the lipids were incubated at room temperature for 45 min in the presence of 7.5% β-ocetyl-sodium and 25 mM dithiothreitol in glycerol. After the reaction, lipids were separated by TLC in chloroform/methanol/acetic acid/HC_O (50:20:15:10:3), and the radioactivity associated with the phosphatidic acid (Phos) fraction was ionized by saponification and measured by scintillation counting. Ceramides and DAG levels were quantitated using external standards and were normalized to phosphate as described previously (32, 33).

**Isolation of the C. neoformans PKC1 Gene—**The C. neoformans PKC1 gene was first identified by blasting the S. cerevisiae homolog in the C. neoformans Genome Project Database of var. grubii serotype A strain JEC21. Three sequences (502511B03.x1, 502488F12.x1, and 502342F06.x1) showed very high homology to the S. cerevisiae PKC1 gene and corresponded to the 3′-region of the gene containing the serine/threonine kinase domain, which is highly conserved among all fungal and human homologs. These sequences were used to design the following primers to amplify a 1589-bp fragment of the PKC1 gene from genomic DNA of C. neoformans var. grubii serotype A strain H99: PKC-antisense-5 (5′-GATT GAA TAT CTA GAT TCC TCT ACA GAT TCA TCA-3′) and PKC-antisense-3 (5′-GTA CTC GAG GTA TCA GGA CAT CCA ATG-3′), which contain EcoRI and XhoI sites (boldface and underlined), respectively. The DNA fragment was then sequenced to confirm the sequence of the upstream and downstream regions of the fragment. To detect the recombinant PKC1 protein, the cDNA of PKC1 from S. cerevisiae was amplified using primers Xo-SeroD (5′-CTA CCT GAA TCA ATG ATC CCA AGA AG-3′) and SeroD-H3 (5′-GAT TAA GCT TCT AGG TGT GTG CAG CCC AAC GAG-3′), which contain XhoI and HindIII sites (boldface and underlined), respectively. The PCR fragment was digested with XhoI and HindIII and cloned into the pmBAD/HisB vector (Invitrogen), placing it in-frame downstream of the ATG start codon and the His and Xpress sequence tags. The resulting plasmid was digested with NotI and HindIII, yielding a fragment containing the ATG start codon, the His and Xpress tags, and PKC1 CDNA sequence. The fragment was blunted and subcloned into HindIII-digested and blunted vector pYES2 (Invitrogen) downstream of the GAL1 promoter, yielding the pYES2/GAL1::Xpress::PKC1 plasmid.

**Expression of Recombinant Pkc1, App1, and ∆C1-Pkc1 Proteins—**To produce the recombinant PKC1 protein, the cDNA of PKC1 from S. cerevisiae was amplified using primers Xo-SeroD (5′-CTA CCT GAA TCA ATG ATC CCA AGA AG-3′) and SeroD-H3 (5′-GAT TAA GCT TCT AGG TGT GTG CAG CCC AAC GAG-3′), which contain XhoI and HindIII sites (boldface and underlined), respectively. The PCR fragment was digested with XhoI and HindIII and cloned into the pmBAD/HisB vector (Invitrogen), placing it in-frame downstream of the ATG start codon and the His and Xpress sequence tags. The resulting plasmid was digested with NotI and HindIII, yielding a fragment containing the ATG start codon, the His and Xpress tags, and PKC1 CDNA sequence. The fragment was blunted and subcloned into HindIII-digested and blunted vector pYES2 (Invitrogen) downstream of the GAL1 promoter, yielding the pYES2/GAL1::Xpress::PKC1 plasmid. To create the recombinant App1 (antiphagocytic protein-1) protein, the pYES2/GAL1::Xpress::PKC1 vector was first digested with SalI and BglII to remove a 1593-bp fragment spanning the sequence corresponding to the C1 domain in pYES2/GAL1::Xpress::PKC1. A 1747-bp DNA fragment downstream of the C1 sequence was amplified by PCR using pYES2/GAL1::Xpress::PKC1 as a template and primers SL1-SeroD (5′-CGT CCT GCA ATG GAT CCC AAC TTA CTC-3′), which contains a SalI site (boldface and underlined)) and SeroD-H3 (5′-GAT TAA GCT TCT AGG TGT GTG CAG CCC AAC GAG-3′). The resulting PCR fragment was digested with SalI and BglII, which yielded a 1177-bp fragment, which was then ligated into the SalI/BglII-restricted pYES2/GAL1::Xpress::PKC1 vector.

**Immunoprecipitation of Pkc1, App1, and ∆C1-Pkc1—**To prepare antibody-bead complexes, 4 μl of anti-Xpress antibody (Invitrogen) were added to 200 μl of protein G-Sepharose 4FF (Amershan Biosciences) in a 1 ml final volume of modified radioimmunoprecipitation assay buffer and incubated for 1 h at 4 °C on a rocking platform. The antibody-bead complexes were washed twice with modified radioimmunoprecipitation assay buffer and centrifuged at 10,000 × g for 1 min at 4 °C. To preclar the cell lysate, an aliquot containing 150 μg of total protein was placed in a 1 ml final volume of modified radioimmunoprecipitation assay buffer with 200 μl of protein G-Sepharose (50% slurry), rocked at 4 °C for 1 h, and then centrifuged for 10,000 × g for 1 min at 4 °C. The preclar supernatant was added to the antibody-bead complexes, and immunoprecipitation was carried out for 16 h at 4 °C on a rocking platform.
onto a 12% SDS-polyacrylamide gel, and separated by SDS-PAGE. Gels were fixed in a 10% methanol and 10% glacial acetic acid solution, rehydrated, and dried on gel blot paper. Radioactively labeled phosphorylated histone was visualized by autoradiography and quantitated using the PhosphorImage system and by ImageQuant analysis (Amersham Biosciences). Specific activity is defined as picomoles of histone phosphorylated per min/mg of lysate, although it may vary according to the level of expression of the recombinant protein. The moles of phosphorylated histone were calculated using a standard curve of the reaction mixture containing both unlabeled ATP and \( {\gamma}^{32}P\)ATP, which had been spotted onto gel blot paper and scanned using a PhosphorImager concurrently with the dried gels. The amount of total lysate corresponding to the amount of recombinant immunoprecipitated Ipc1 used in the assay was determined by subjecting an aliquot of the immunoprecipitate with a standard curve of the total lysate containing the recombinant protein to Western blotting. The reaction time for the kinase assay and the concentration of immunoprecipitated protein used were chosen after performing a time course and protein titration to ensure that the measurements were within the linear range of kinase activity.

**Ip1 Activity and Western Blotting**—The Ip1 activity of the C. neoformans wild-type and GAL7::IPC1 strains grown in 0.1% galactose and 2% raffinose was measured as described previously (23) and quantitated using a PhosphorImager and by ImageQuant analysis. The specific activity of Ip1 is defined as picomoles of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-inositol phosphorylceramide produced per min/mg of total cell lysate per protein. The quantity of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-inositol phosphorylceramide produced was determined using a standard curve of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-C, which was subjected to TLC alongside the experimental samples. For Western blot analysis, samples were loaded onto a 6% SDS-polyacrylamide gel, separated by electrophoresis, and blotted onto nitrocellulose membrane. The membrane was blocked in 5% nonfat milk/1× PBST (phosphate-buffered saline and 0.1% Tween 20). The anti-X-Pers antibody (Invitrogen) was used at 1:2500 dilution in 3% bovine serum albumin/1× PBST. The secondary antibody used was horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The membrane was washed with 1× PBS, treated with ECL Western blot detection reagents (Amer sham Biosciences), and exposed to Kodak BioMax film.

**Laccase Activity**—Laccase activity was assayed as described previously with minor modifications (37, 38). Cells were inoculated from fresh cultures in YPD medium into YP broth with 2% glucose or raffinose (repressing conditions). Laccase activity and melanin production by the transformants were also analyzed as described above.

**Ip1 Modulation**—To ensure repression of the GAL7::IPC1 strain in the presence of galactose and less melanin in the presence of raffinose. No significant difference was observed in melanin production by the wild-type strain grown in galactose or raffinose. Ip1 activity increased when the GAL7::IPC1 strain was grown in galactose and decreased when the GAL7::IPC1 strain was grown in raffinose compared with the wild-type strain. Data are representative of at least three independent experiments.

**RESULTS**

**Ip1 Regulates Melanin Production by C. neoformans**—In previous studies, we generated a C. neoformans GAL7::IPC1 strain in which the IPC1 gene is regulated by the galactose-inducible GAL7 promoter (23). This strain allows up-regulation of IPC1 by growing cells in galactose medium (inducing conditions) and down-regulation of IPC1 by growing cells in glucose or raffinose medium (repressing conditions). Using the GAL7::IPC1 strain, we showed that Ip1 regulates the activity of laccase, the melanin-producing enzyme of C. neoformans, as measured by an in vitro assay (23). Thus, we wondered whether Ip1 modulation would affect the production of melanin pigment in vivo (growing cells). The C. neoformans wild-type (H99)

University Medical Center) using primers XB-NAT-F (5′-CTA ATC TAG ACC GAT GTG TGG AGA GGC GGC G-3′) and XB-NAT-R (5′-CGG GTG TAG AGA AGA GAT GTA GAA ACT AGC TTC C-3′), which contain XbaI sites (boldface and underlined); subcloned into the pCR2.1-TOPO vector (Invitrogen); excised by digestion with XbaI; and blunted. Plasmid pUR5/GAL7::PKC1-AS was digested with SacI and XbaI, blunted, dephosphorylated, and ligated with the NAT1 fragment. This pNAT1/GAL7::PKC1-AS construct was then transformed into both C. neoformans wild-type and GAL7::IPC1 strains by biolistic transformation (42) to produce the C. neoformans IPC1/IPC1-AS and GAL7::IPC1/PKC1-AS strains, respectively. Transformants were screened for homologous recombination by screening for resistance to the selectable marker. Three stable nourseothricin-resistant transformants of each strain (IPC1/IPC1-AS, transformants 16, 18, and 19) and GAL7::IPC1-AS, transformants 5, 27, and 31) were chosen randomly and grown in YP broth containing 2% galactose, glucose, or raffinose for 24 h at 30 °C. Total RNA was extracted from each culture and used for analysis of PKC1 mRNA levels by reverse transcription-PCR using primers PKC-5 (5′-GAG GGA GAG GAA AAA ATC-3′) and PKC-antisense-3 (5′-GTA CTC GAG CAT GTA GAA ACT AGC TTC C-3′). Laccase activity and melanin production by the transformants were also analyzed as described above.

**Statistical Analysis**—Statistical analysis was performed using Student’s t test.

**RESULTS**

**Ip1 Regulates Melanin Production by C. neoformans**—In previous studies, we generated a C. neoformans GAL7::IPC1 strain in which the IPC1 gene is regulated by the galactose-inducible GAL7 promoter (23). This strain allows up-regulation of IPC1 by growing cells in galactose medium (inducing conditions) and down-regulation of IPC1 by growing cells in glucose or raffinose medium (repressing conditions). Using the GAL7::IPC1 strain, we showed that Ip1 regulates the activity of laccase, the melanin-producing enzyme of C. neoformans, as measured by an in vitro assay (23). Thus, we wondered whether Ip1 modulation would affect the production of melanin pigment in vivo (growing cells). The C. neoformans wild-type (H99)

**REFERENCES**

1. Leupold, D. A., and Brown, D. J. (1997) J. Bacteriol. 179, 7551–7557.
GAL7::IPC1 strains were grown on agar containing L-DOPA, which is a substrate for laccase, with either galactose or raffinose. The production of melanin was visualized by the formation of the characteristic brown pigment. As shown in Fig. 1A, the GAL7::IPC1 strain produced more melanin compared with the wild-type strain on L-DOPA/galactose agar, whereas it produced less melanin compared with the wild-type strain on L-DOPA/raffinose agar. No significant difference in melanin production was detected with the wild-type strain when grown in galactose or raffinose. We confirmed that, under these experimental conditions, Ipc1 activity in the GAL7::IPC1 strain was up- and down-regulated, respectively, compared with the wild-type strain (Fig. 1B).

Ipc1 Regulates DAG and Phytoceramide Levels in C. neoformans—We next investigated the mechanisms by which Ipc1 may regulate melanin formation in C. neoformans. Because mammalian melanogenesis is suggested to be dependent on a DAG-PKC pathway and because Ipc1 catalyzes the production of DAG, we wondered whether Ipc1 may control the activation of fungal Pkc1 through the modulation of DAG. First, we determined whether Ipc1 regulates the level of DAG in C. neoformans. The wild-type and GAL7::IPC1 strains were grown under inducing and repressing conditions, and total DAG levels in each sample were measured by in vitro DAG kinase assay. As shown in Fig. 2A, down-regulation of IPC1 decreased DAG levels, and up-regulation of IPC1 increased DAG levels in the GAL7::IPC1 strain compared with the wild-type (WT) strain. *, p < 0.02. B, down-regulation of IPC1 caused a buildup of cellular phytoceramide. *, p < 0.02. Data are shown as picomoles of DAG or phytoceramide/nmol of P_i.
Isolation and Expression of the PKC1 Gene—Because the above data demonstrated that Ipc1 regulates the levels of DAG and phytoceramide (an activator and inhibitor of mammalian PKC, respectively) (43, 44), we next wondered whether these two lipids regulate the activity of C. neoformans Pkc1. To measure Pkc1 activity, it was necessary to produce and isolate the recombinant Pkc1 protein.

First, the C. neoformans PKC1 gene was identified and isolated from genomic DNA and cDNA libraries of serotype A strain H99 and serotype D strain B3501. Strands of the open reading frame and 5′- and 3′-untranslated regions were sequenced, revealing that the PKC1 gene is 3784 base pairs in length with 10 intronic sequences, yielding a predicted protein of 1086 amino acids (GenBank™/EBI Data Bank accession numbers AY373758 and AY373759). Compared with mammalian PKCs, C. neoformans Pkc1 was most similar to the DAG-dependent atypical mammalian PKCs, lack two or more of these key residues (47). Based on these criteria, the C1A domain of C. neoformans Pkc1 was classified as a typical domain because it has conservation of the three DAG-binding residues as well as four of the five hydrophobic residues (Fig. 3B). In contrast, the C1A and C1B domains of the C. albicans Pkc1 proteins lack the majority of these important residues (Fig. 3B) and are therefore classified as atypical (47, 50). The C. neoformans C1A domain was 35.4% similar to the PKCδ C1A domain, whereas the S. cerevisiae and C. albicans C1A domains were 27.7 and 29.8% similar to the PKCδ C1A domain, respectively.

To express the recombinant Pkc1 protein, the PKC1 cDNA was fused to the Xpress epitope tag under the control of the galactose-inducible GAL1 promoter in the pYES2 expression vector. The C1 domain of mammalian and fungal PKCs consists of two tandem DAG-binding domains designated C1A and C1B (47), each of which represents a zinc finger motif due to a conserved pattern of histidine and cysteine residues (shown in boldface in Fig. 3B) (48, 49). It has been suggested that only one of these zinc finger motifs (C1A or C1B) is required for the activation of PKC by DAG (47). These C1A and C1B motifs of mammalian and fungal PKCs can be classified as “typical” or “atypical” to refer to C1 domains that bind or do not bind DAG, respectively. Typical C1 domains, found in the DAG-dependent classical and novel PKC isoforms, contain three consensus residues (Pro, Gly, and Gln) that maintain the structure of the DAG-binding pocket as well as five consensus residues at positions 8, 13, 20, 22, and 24, which compose a hydrophobic wall around the binding site, as illustrated in Fig. 3B for the C1A motif (47).

Using the above criteria, the C1A domain of C. neoformans Pkc1 was classified as a typical domain because it has conservation of the three DAG-binding residues as well as four of the five hydrophobic residues (Fig. 3B). In contrast, the C1A and C1B domains of the C. neoformans Pkc1 proteins lack the majority of these important residues (Fig. 3B) and are therefore classified as atypical (47, 50). The C. neoformans C1A domain was 35.4% similar to the PKCδ C1A domain, whereas the S. cerevisiae and C. albicans C1A domains were 27.7 and 29.8% similar to the PKCδ C1A domain, respectively.

To express the recombinant Pkc1 protein, the PKC1 cDNA was fused to the Xpress epitope tag under the control of the galactose-inducible GAL1 promoter in the pYES2 expression vector. The C1 domain of mammalian and fungal PKCs consists of two tandem DAG-binding domains designated C1A and C1B (47), each of which represents a zinc finger motif due to a conserved pattern of histidine and cysteine residues (shown in boldface in Fig. 3B) (48, 49). It has been suggested that only one of these zinc finger motifs (C1A or C1B) is required for the activation of PKC by DAG (47). These C1A and C1B motifs of mammalian and fungal PKCs can be classified as “typical” or “atypical” to refer to C1 domains that bind or do not bind DAG, respectively. Typical C1 domains, found in the DAG-dependent classical and novel PKC isoforms, contain three consensus residues (Pro, Gly, and Gln) that maintain the structure of the DAG-binding pocket as well as five consensus residues at positions 8, 13, 20, 22, and 24, which compose a hydrophobic wall around the binding site, as illustrated in Fig. 3B for the C1A motif (47).

Atypical C1 domains, found in the DAG-independent atypical mammalian PKCs, lack two or more of these key residues (47). Based on these criteria, the C1A domain of C. neoformans Pkc1 was classified as a typical domain because it has conservation of the three DAG-binding residues as well as four of the five hydrophobic residues (Fig. 3B). In contrast, the C1A and C1B domains of the C. neoformans Pkc1 proteins lack the majority of these important residues (Fig. 3B) and are therefore classified as atypical (47, 50). The C. neoformans C1A domain was 35.4% similar to the PKCδ C1A domain, whereas the S. cerevisiae and C. albicans C1A domains were 27.7 and 29.8% similar to the PKCδ C1A domain, respectively.

To express the recombinant Pkc1 protein, the PKC1 cDNA was fused to the Xpress epitope tag under the control of the galactose-inducible GAL1 promoter in the pYES2 expression vector. The C1 domain of mammalian and fungal PKCs consists of two tandem DAG-binding domains designated C1A and C1B (47), each of which represents a zinc finger motif due to a conserved pattern of histidine and cysteine residues (shown in boldface in Fig. 3B) (48, 49). It has been suggested that only one of these zinc finger motifs (C1A or C1B) is required for the activation of PKC by DAG (47). These C1A and C1B motifs of mammalian and fungal PKCs can be classified as “typical” or “atypical” to refer to C1 domains that bind or do not bind DAG, respectively. Typical C1 domains, found in the DAG-dependent classical and novel PKC isoforms, contain three consensus residues (Pro, Gly, and Gln) that maintain the structure of the DAG-binding pocket as well as five consensus residues at positions 8, 13, 20, 22, and 24, which compose a hydrophobic wall around the binding site, as illustrated in Fig. 3B for the C1A motif (47).

Atypical C1 domains, found in the DAG-independent atypical mammalian PKCs, lack two or more of these key residues (47). Based on these criteria, the C1A domain of C. neoformans Pkc1 was classified as a typical domain because it has conservation of the three DAG-binding residues as well as four of the five hydrophobic residues (Fig. 3B). In contrast, the C1A and C1B domains of the C. neoformans Pkc1 proteins lack the majority of these important residues (Fig. 3B) and are therefore classified as atypical (47, 50). The C. neoformans C1A domain was 35.4% similar to the PKCδ C1A domain, whereas the S. cerevisiae and C. albicans C1A domains were 27.7 and 29.8% similar to the PKCδ C1A domain, respectively.
Experimental Procedures

B. S. cerevisiae Munoprecipitated from the results from Western blot analysis of C1-Pkc1/H9004 analysis using anti-Xpress antibody. As depicted in Fig. 3, DAG.

A. pYES2/GAL1::Xpress::PKC1

Next, the effect of ceramides, which inhibit certain mammalian PKC isoforms (43, 44), on Pkc1 activity was evaluated. As shown in Fig. 4C, the short-chain ceramide analogs C6-phytoceramide and C4-ceramide decreased Pkc1 activity by ~50% compared with the untreated sample.

Deletion of the C1 Domain Abolishes the Activation of Pkc1 by DAG—To investigate a possible mechanism by which DAG activates Pkc1, we focused on the putative DAG-binding domain (C1), which was deleted from the pYES2/GAL1::Xpress::PKC1 expression vector (Fig. 5A). The resulting pYES2/GAL1::Xpress::PKC1 construct was transformed into the recipient S. cerevisiae JK9-3Da strain. Transformants were screened by Western blot analysis using anti-Xpress antibody. As depicted in Fig. 3D, under repressing conditions (galactose), a single band was detected at ~120 kDa, the predicted size of Pkc1 including the Xpress epitope tag, whereas no expression was detected under derepressing conditions (glucose). The Pkc1 protein was immunoprecipitated from the total cell lysate using anti-Xpress antibody conjugated to protein G-Sepharose beads (Fig. 3D). The isolated recombinant C. neoformans Pkc1 protein was then used for in vitro kinase assays.

Pkc1 Is Activated by DAG and Inhibited by Phytoceramide—To determine whether the activity of Pkc1 may be regulated by DAG and/or phytoceramide, kinase assays were performed to test the ability of recombinant C. neoformans Pkc1 to phosphorylate histone substrate in the presence or absence of DAG or phytoceramide delivered in Triton X-100/lipid mixed micelles. In the absence of lipids, the specific activity of Pkc1 was 31.5 pmol/min/mg. In the presence of the DAG subtypes dipalmitoylglycerol and dimyristoylglycerol, Pkc1 activity was increased by 1.7- and 2-fold, respectively (Fig. 4A). A third DAG subtype, palmitoyloleoylglycerol, caused a 1.5-fold increase in Pkc1 activity (data not shown). Interestingly, Pkc1 activity also increased in the presence of phosphatidylserine (Fig. 4A), a known lipid cofactor of mammalian PKC.

To ensure that the kinase activity detected was specific to Pkc1, a different Xpress-tagged protein from C. neoformans (App1) was immunoprecipitated from cell lysates of S. cerevisiae strain JK9-3Da expressing the pYES2/GAL1::Xpress::APP1 vector and used in the same kinase assay. As shown in Fig. 4B, App1 did not cause any notable phosphorylation of histone in the presence or absence of DAG, as expected. The specific activity of App1 in the absence of lipid was estimated at 7.1 pmol/min/mg.

Next, the effect of ceramides, which inhibit certain mammalian PKC isoforms (43, 44), on Pkc1 activity was evaluated. As shown in Fig. 4C, the short-chain ceramide analogs C6-phytoceramide and C4-ceramide decreased Pkc1 activity by ~50% compared with the untreated sample.

Inhibition of Pkc1 Inhibits Laccase Activity and Melanin Production—Because the above data suggested that Ipc1 may regulate Pkc1 through the production of DAG and because DAG-PKC signaling may control melanogenesis in mammalian models (28, 29), we investigated whether Pkc1 plays a role in the regulation of melanin formation by C. neoformans. First, a pharmacological approach was used in which laccase activity
was measured in *C. neoformans* wild-type and GAL7::IPC1 cells grown in galactose or glucose upon treatment with mammalian serine/threonine kinase inhibitors such as staurosporine, bisindolylmaleimide I, and calphostin C. Staurosporine is a nonspecific inhibitor of PKC that targets the ATP-binding site (51). As shown in Fig. 6A, treatment of *C. neoformans* with increasing concentrations of staurosporine (1, 5, and 10 μM) caused a dose-dependent decrease in laccase activity. At the highest concentration of staurosporine (10 μM), there was an ~65% decrease in laccase activity in the wild-type strain in both galactose and glucose compared with vehicle controls. The GAL7::IPC1 strain grown in galactose showed a more modest, but significant 33% decrease in laccase activity. The GAL7::IPC1 strain grown in glucose exhibited the most inhibition at 78% (Fig. 6A).

Bisindolylmaleimide I is specifically directed against the ATP-binding site of PKC (52). Like staurosporine, bisindolylmaleimide I induced a dose-dependent decrease in laccase activity (Fig. 6B). However, bisindolylmaleimide I was more potent than staurosporine. Wild-type cells treated with 10 μM bisindolylmaleimide I showed a 72% decrease in laccase activity. GAL7::IPC1 cells in galactose showed a 48% decrease, and GAL7::IPC1 cells in glucose had a decrease of 84% (Fig. 6B).

Calphostin C is a specific inhibitor of PKC that blocks the binding of DAG to PKC (53). As illustrated in Fig. 6C, treatment with calphostin C also caused a dose-dependent decrease in laccase activity and proved to be more potent than staurosporine and bisindolylmaleimide I in the inhibition of laccase activity. Indeed, the laccase activity of the wild-type strain decreased by 83% upon treatment with 10 μM calphostin C, whereas the laccase activity of the GAL7::IPC1 strain decreased by ~85% (Fig. 6C).

To determine whether these decreases in laccase activity were specific to inhibition of a serine/threonine kinase, the *C. neoformans* wild-type and GAL7::IPC1 strains grown in glucose or galactose were treated with tyrosine kinase inhibitors such as genistein and herbimycin A at the same concentrations (1, 5, and 10 μM). As shown in Fig. 6D, genistein treatment did not affect laccase activity. Similar results were obtained with herbimycin A (data not shown).

To verify that the above inhibitors were nontoxic to *C. neoformans*, treated cells were tested for cell viability. There was no significant change in the number of colony-forming units of *C. neoformans* cells upon treatment with staurosporine, bisindolylmaleimide I, calphostin C, genistein, or herbimycin A, indicating that these inhibitors are nontoxic to the cells at the concentrations used (data not shown).

Next, to confirm the direct involvement of Pkc1 in the regulation of melanin production by *C. neoformans*, Pkc1 was specifically down-regulated by expression of an antisense PKC1 transcript. A PCR fragment corresponding to the 3′-region of the PKC1 gene containing the serine/threonine kinase domain (see “Experimental Procedures”) was used to create the pNAT1/GAL7::PKC1-AS construct (Fig. 7A). This construct was transformed into the *C. neoformans* wild-type (H99) and GAL7::IPC1 strains. Nourseothricin-resistant transformants of each strain were isolated and named IPC1/PKC1-AS and GAL7::IPC1/PKC1-AS strains, respectively. Next, three inde-
pendent transformants for the IPC1/PKC1-AS strain (transformants 16, 18, and 19) and the GAL7::IPC1/PKC1-AS strain (transformants 5, 27, and 31) were randomly selected and grown in galactose for expression of antisense PKC1 and in glucose or raffinose as a control. As illustrated in Fig. 7 (B and C), expression of antisense PKC1 decreased laccase activity in the three independent IPC1/PKC1-AS transformants 16, 18, and 19 compared with the parental wild-type (WT) strain. C, expression of antisense PKC1 in the three independent GAL7::IPC1/PKC1-AS transformants 5, 27, and 31 decreased laccase activity compared with the parental GAL7::IPC1 strain. D, induction of antisense PKC1 decreased melanin production by C. neoformans on L-DOPA/galactose agar plates. E, induction of antisense PKC1 blocked the up-regulation of melanin production by Ipcl. Data are representative of at least three separate experiments.

**DISCUSSION**

In this study, the signaling pathway by which the sphingolipid enzyme Ipcl regulates melanin production by C. neoformans was investigated. The results suggest that the sphingolipid pathway controls the activation of Pkc1 via Ipcl through the regulation of DAG and phytoceramide. Additionally, evidence is provided that the C1 domain of Pkc1 is required for activation by DAG. Finally, the results demonstrate that Pkc1 is essential for melanin formation in C. neoformans. Taken together, these results provide evidence for a novel signaling pathway (Ipcl-DAG/phytoceramide-Pkc1) that plays a crucial role in melanin production and in the pathogenicity of C. neoformans.
Relatively little is known about the regulatory pathways leading to the formation of melanin in *C. neoformans*. Recent studies show that the Gα protein-adenyl cyclase-cAMP-protein kinase A signaling cascade may regulate melanin formation (38, 54, 55). However, studies in mammalian cells suggest that melanin production is regulated by a DAG-PKC mechanism (28, 29, 56). Here, we extend our previous observations that *C. neoformans* Ipc1 controls the formation of melanin (23) and provide evidence for a key role for the DAG-Pkc1 pathway.

We found that modulation of Ipc1 causes significant changes in the level of DAG in *C. neoformans*. These changes were not dramatic, but a possible explanation may involve the modulation of subspecies of DAG by Ipc1. An interesting study by Schneiter et al. (57) showed that the distribution of phosphatidylglycerol (substrate for Ipc1) within fungal cells is heterogeneous. In particular, using mass spectrometry, they showed that different phosphatidylglycerol subspecies, defined by the composition of fatty acids in position 1 or 2 of the glycerol backbone, are localized in different subcellular compartments (i.e., Golgi versus plasma membrane). Because the Ipc1 enzyme is localized in the Golgi (58), it may be exposed to a distinct population of phosphatidylglycerol subspecies. As a consequence, Ipc1 may regulate the production of only one or a few DAG subspecies, which may represent a small subset of the total DAG found in the cell. Another possibility could be the selectivity of Ipc1 for phosphatidylglycerol subspecies, as observed for other enzymatic reactions in yeast and mammalian cells, such as the acylation of glycosylphosphatidylinositols in *C. neoformans* (59) and the hydrolytic activity of phospholipase C (60). Importantly, the hypothesis of species selectivity is strengthened by the observation that DAG subspecies differentially activated Pkc1. In particular, dimyristoylglycerol was the most potent activator, followed closely by dipalmitoylglycerol (Fig. 4A). Palmitoyloleoylglycerol also activated Pkc1, but less significantly compared with the other two DAG subspecies (data not shown).

Interestingly, the sensitivity of *C. neoformans* Pkc1 to DAG may be due to the structure of its C1 domain, which appears to be that of a typical DAG-binding domain. In contrast, the C1 domains of *S. cerevisiae* and *C. albicans* are classified as atypical or DAG-insensitive. Studies using recombinant Pkc1 proteins from *S. cerevisiae* and *C. albicans* indicate that these serine/threonine protein kinases are not activated by DAG in vitro (19–21), although there is some indirect evidence that Pkc1 from *S. cerevisiae* is activated by DAG (17, 18, 61, 62). We must note that the conflicting results concerning DAG activation of the fungal Pkc1 proteins could be attributed to the different experimental conditions used to assess kinase activity. Unlike the other studies, this study used a Triton X-100/lipid mixed micelle system for the delivery of DAG and other lipids. Additionally, because only certain DAG subspecies may be able to activate Pkc1, as discussed above, perhaps the choice of DAG subspecies accounts for the different outcomes, as already suggested by Marini et al. (62). On the other hand, in *C. neoformans*, the DAG-Pkc1 signaling mechanism may be specifically used by this pathogenic fungus to produce melanin, which is not produced by *S. cerevisiae* or *C. albicans*. Thus, the fundamental differences in both C1 domain structure and cellular function among the fungal Pkc1 proteins indicate that Pkc1 from *C. neoformans* is truly a distinct class of fungal protein kinase C from that of *S. cerevisiae* and *C. albicans*.

The regulation of Pkc1 by Ipc1 may be mediated not only by the production of DAG, but also by the modulation of phytoceramide. Down-regulation of Ipc1 in vitro caused a significant buildup of phytoceramide in *C. neoformans* cells (Fig. 2B), and both phytoceramide and ceramide inhibited Pkc1 activity in vitro (Fig. 4C). Studies in mammalian cells suggest that ceramide may inhibit PKC directly by binding at the C1 domain and thereby competing with DAG (43) or indirectly through the activation of a phosphatase that dephosphorylates PKC (44). Because phytoceramide alone can inhibit *C. neoformans* Pkc1 in the kinase assay, a direct mechanism of action is likely, at least in this particular fungal system. However, phytoceramide was still able to inhibit Pkc1 even when the C1 domain was removed (Fig. 5C), although this inhibition was attenuated, suggesting that phytoceramide binds at a different regulatory domain or is able to regulate Pkc1 through more than one domain. These observations support a model in which Ipc1 functions as a molecular switch for the activation of Pkc1 in *C. neoformans* by regulating at the same time and in opposite directions the levels of DAG (activator) and phytoceramide (inhibitor). Such possibilities clearly warrant further investigation.

The observation that up-regulation of Ipc1 did not decrease phytoceramide below the level found in wild-type cells under the same conditions (Fig. 2B) is intriguing. This result could be explained by a compensatory up-regulation of the sphingolipid enzymes upstream of Ipc1 to maintain a constant level of phytoceramide. Indeed, the mRNA level of the *LCB2* (long-chain base 2) gene, which encodes the catalytic subunit of Spt1 (serine palmitoyltransferase-1), the first enzyme in sphingolipid synthesis, was increased when Ipc1 was up-regulated in the *GAL7::IPC1* strain. The activation of the Spt1 enzyme upon up-regulation of Ipc1 may account for an increase in the

---

4 M. Del Poeta, unpublished data.
de novo synthesis of phytoceramide, thereby maintaining a constant level of intracellular phytoceramide.

In conclusion, our results establish a novel role for the sphingolipid enzyme Ipc1 in the regulation of fungal Pkc1. Ipc1 has previously been recognized as a promising target for antifungals because it is a fungus-specific enzyme (14). Now, because the newly defined Ipc1-Pkc1 pathway appears to be crucial for melanin formation in C. neoformans, targeting Ipc1 and its downstream effectors for the development of new antifungal drugs is even more attractive (Fig. 8). Importantly, with the demonstration that Ipc1 activates Pkc1 through the formation of DAG, this study provides the first biochemical evidence that a link between sphingolipid-derived DAG and protein kinase C activity exists and could represent a paradigm that is applicable to mammalian systems.

Acknowledgments—We thank Drs. Peter Williamson, Lina Obeid, Jeffrey Jones, and Kevin Becker for helpful discussions; Daniel Taraskiewicz for technical assistance; Drs. Alicja Bielawska, Jacek Bielawski, and Zdzislaw Szulc (Medical University of South Carolina Lipidomics Core Facility) for synthesis of ceramides; and LuAnne Harley for help in the preparation of this manuscript. We give special thanks to Drs. Gary Cox and John Perfect for the generous gifts of the antisense and NAT71 plasmids and to Dr. Steven Kubalak and the members of his laboratory for help with photography.

REFERENCES
1. Hannun, Y. A., Luberto, C., and Argraves, K. M. (2001) Biochemistry 40, 4893–4903
2. Patton, J. L., Srinivasan, B., Dickson, R. C., and Lester, R. L. (1992) J. Biol. Chem. 174, 7180–7184
3. Jenkins, G. M., Richards, A., Wahl, T. M., Obeid, L., and Hannun, Y. (1997) J. Biol. Chem. 272, 35256–35272
4. Munn, A. L., and Riezman, H. (1994) J. Cell Biol. 127, 373–386
5. Zanolin, B., Friel, S., Funato, K., Sutterlin, C., Stevenson, B. J., and Riezman, H. (2000) EMBO J. 19, 2824–2833
6. Obeid, L. M., Okamoto, Y., and Man, C. (2002) Biochim. Biophys. Acta 1585, 163–171
7. Ullman, D., and Radin, N. S. (1974) J. Biol. Chem. 249, 1506–1512
8. Voelker, D. R., and Kennedy, E. P. (1982) Biochemistry 21, 2753–2759
9. Bernert, J. T., Jr., and Ullman, M. D. (1981) J. Cell Biol. 85, 275–282
10. Voelker, D. R., and Kennedy, E. P. (1982) Biochemistry 21, 2753–2759
11. Ullman, D., and Radin, N. S. (1974) J. Biol. Chem. 249, 1506–1512
12. Voelker, D. R., and Kennedy, E. P. (1982) Biochemistry 21, 2753–2759
13. Bernert, J. T., Jr., and Ullman, M. D. (1981) Biochim. Biophys. Acta 666, 99–109
14. Marggraf, W. D., Anderer, F. A., and Kanfer, J. N. (1981) Biochim. Biophys. Acta 664, 61–73
15. Marggraf, W. D., Zettani, R., Anderer, F. A., and Kanfer, J. N. (1982) Biochim. Biophys. Acta 710, 314–323
16. Merril, A. H., Jr., and Jones, D. D. (1990) Biochim. Biophys. Acta 1044, 1–12
17. Hatch, G. M., and Van Der, F. E. (1992) J. Biol. Chem. 267, 24449–24451
18. Nagie, M. M., Nagie, E., Balsbergterger, J. A., Wells, G. B., Lester, R. L., and Dickson, R. C. (1997) J. Biol. Chem. 272, 8969–8971
19. Luberto, C., Yoo, D. S., Suidan, H. S., Bartoli, G. M., and Hannun, Y. (2000) J. Biol. Chem. 275, 14760–14766
20. Signorelli, P., Luberto, C., and Hannun, Y. A. (2001) FASEB J. 15, 2401–2414
21. Ogita, K., Miyamoto, S., Koide, H., Ita, W., Oka, M., Ando, K., Kishimoto, A., Ieda, K., Fukami, Y., and Nishizuka, Y. (1996) Proc. Natl. Acad. Sci. U.S.A. 87, 5011–5015
22. Simon, A. J., Milner, Y., Saville, S. P., Dvor, A., Molyb-Rosen, D., and Orr, E. (1993) Proc. R. Soc. Lond. B Biol. Sci. 243, 165–171
23. Watanabe, M., Chen, C. Y., and Levin, D. E. (1994) J. Biol. Chem. 269, 16829–16836
24. Paravicini, G., Mendoza, A., Antonsson, B., Cooper, M., Losberger, C., and Payton, M. A. (1996) Yeast 12, 741–756
25. Antonsson, B., Montessuit, S., Friedli, L., Payton, M. A., and Paravicini, G. (1994) J. Biol. Chem. 269, 16821–16825
26. Casadevall, A., and Perfect, J. R. (1998) Cryptococcus neoformans, American Society for Microbiology, Washington, D. C.
The Sphingolipid Pathway Regulates Pkc1 through the Formation of Diacylglycerol in Cryptococcus neoformans

Lena J. Heung, Chiara Luberto, Allyson Plowden, Yusuf A. Hannun and Maurizio Del Poeta

J. Biol. Chem. 2004, 279:21144-21153. doi: 10.1074/jbc.M312995200 originally published online March 10, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M312995200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 33 of which can be accessed free at http://www.jbc.org/content/279/20/21144.full.html#ref-list-1