APP1 Transcription Is Regulated by Inositol-phosphorylceramide Synthase 1-Diacylglycerol Pathway and Is Controlled by ATF2 Transcription Factor in Cryptococcus neoformans*

Lydia Mare, Roberta Iatta, Maria Teresa Montagna, Chiara Luberto, 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 and the Department of Internal and Public Medicine, University of Bari, 70124 Bari, Italy

Inositol-phosphorylceramide synthase 1 (Ipc1) is a fungal-specific enzyme that regulates the level of two bioactive molecules, phytoceramide and diacylglycerol (DAG). In previous studies, we demonstrated that Ipc1 regulates the expression of the antiphagocytic protein 1 (App1), a novel fungal factor involved in pathogenicity of Cryptococcus neoformans. Here, we investigated the molecular mechanism by which Ipc1 regulates App1. To this end, the APP1 promoter was fused to the firefly luciferase gene in the C. neoformans GAL7:IPC1 strain, in which the Ipc1 expression can be modulated, and found that the luciferase activity was indeed regulated when Ipc1 was modulated. Next, using the luciferase reporter assay in both C. neoformans wild-type and GAL7:IPC1 strains, we investigated the role of DAG and sphingolipids in the activation of the APP1 promoter and found that treatment with 1,2-dioctanoylglycerol does increase APP1 transcription, whereas treatment with phytosphingosine or ceramides does not. Two putative consensus sequences were found in the APP1 promoter and found that treatment with 1,2-dioctanoylglycerol does increase APP1 transcription, whereas treatment with phytosphingosine or ceramides does not. Two putative consensus sequences were found in the APP1 promoter and treatment with DAG activates luciferase in a dose- and time-dependent manner only when the luciferase gene is under the control of the APP1 promoter.

APP1 transcription is regulated by Inositol-phosphorylceramide synthase 1-Diacylglycerol Pathway and is controlled by ATF2 transcription factor.
AP-2 causes a significant increase of APP1 transcription, suggesting that this consensus sequence acts as a negative regulator. Finally, we identified and deleted the putative C. neoformans ATF2 gene by homologous recombination and found that loss of Atf2 abrogates luciferase activation driven by the APP1 promoter and regulated by Ipc1 or DAG. Thus, these studies suggest that APP1 transcription is under the control of the Ipc1-DAG pathway through the Atf2 transcription factor and two consensus sequences (AP-2 and ATF) present in the APP1 promoter.

MATERIALS AND METHODS

Strains, Growth Media, and Reagents—C. neoformans var. grubii serotype A strain H99 and derivative mutants used in this study are illustrated in TABLE ONE. The strains were routinely grown in yeast extract/peptone/dextrose (YPD) medium. Yeast extract peptone (YP) supplemented with 20 g/liter glucose or 20 g/liter galactose was used to down- or up-regulate the expression of IPC1 gene, respectively. Nourseothricin (Werner BioAgents, Germany) at a concentration of 100 μg/ml was added to YPD plates for selection of the IPC1/APP1::LUC, GAL7::IPC1/APP1::LUC, IPC1::ACT1::LUC, GAL7::IPC1::S'UTR: LUC, and IPC1/366::LUC strains, as indicated. Hygromycin B (Calbiochem, San Diego, CA) at a concentration of 200 units/ml was added to YPD plates for selection of IPC1/APP1::LUC/Δatf2 and GAL7::IPC1/ APP1::LUC/Δatf2 strains. C. neoformans strains carrying episomal plasmids (numbers 8–13, TABLE ONE), were routinely grown onto YPD medium containing 200 units/ml of hygromycin B.

Nuclear Run-on Assay—The nuclear run-on assay was performed according to Hirayoshi and Lis (17). Briefly, C. neoformans IPC1 (WT) and GAL7::IPC1 strains were grown on YP-glucose medium in a shaker incubator for 24 h at 30 °C. Cells were washed 3 times in sterile-distilled water (SDW) and then incubated in YNB broth containing 2% glucose or 2% galactose for 24 h at 30 °C. Cells were collected at 2800 × g, washed 3 times in 0.5 M NaCl, 50 mM EDTA, suspended in 9.5 ml of SDW containing 0.5 ml of -mercaptoethanol, and incubated at 37 °C for 1 h. The cell pellet was then collected at 2800 × g, suspended in 4 ml of spheroplastic solution (1 M sorbitol, 0.1 M Na citrate, pH 5.8, 0.01 M EDTA), and placed on ice for 10 min. Then, 1 ml of spheroplastic solution containing 10 mg of lysing enzyme (Sigma number L-1412) was added, and cells were incubated at 37 °C for 1 h. Next, 2.5 × 107 cells were harvested at 3000 × g for 6 min at room temperature and washed with TMN buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl2). Cells were suspended and kept on ice for 15 min in Sarkosyl solution (0.95 ml of SDW, 0.05 ml of 10% (w/w) N-lauroylsarcosine (Sigma L-9150)). After centrifugation at 2500 × g for 1 min at 4 °C, the cells were suspended in 100 μl of reaction buffer (50 mM Tris-HCL, pH 7.9, 100 mM KCl, 5 mM MgCl2, 1 mM MnCl2, 2 mM dithiothreitol, 0.5 mM ATP, GTP, CTP, 100 μCi of [α-32P]UTP, and 1 units of RNasin). The
reaction was incubated at 25 °C for 8 min and terminated by the addition of 1 μl of 1 mg/ml α-amanitin and 20 μl of 50 mg/ml DNase I. The mixture was incubated for 10 min at 30 °C and then an equal volume of stop buffer (~120 μl) containing 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 2% SDS, and 200 μg/μl proteinase K was added and the mixture incubated at 42 °C for 30 min. The labeled mRNA was extracted using phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma number P-3803) and precipitated with ethanol. The labeled mRNA was suspended in 100 μl of diethyl pyrocarbonate water and added to the hybridization chamber containing nitro membranes in which actin, IPC1, and APP1 cDNA were previously immobilized using a slot blot apparatus. Before adding the labeled mRNA, the nitro membranes were pre-hybridized for 3 h at 58 °C in hybridization solution (10% dextran sulfate, 1% SDS, 50% formamide, 6× standard saline citrate (SSC)). Once the labeled mRNA was added, the nitro membranes were hybridized at 58 °C for 16 h. After hybridization, the membranes were washed 3 times at 58 °C for 10 min each in 2× SSC and 1% SDS, 3 times at 68 °C for 10 min each in 2× SSC and 1% SDS, and 3 times at 68 °C for 10 min each in 0.1× SSC and 1% SDS. Membranes were then air-dried and exposed to phosphor screen at room temperature for 6 days. Each band was quantified by a phosphoimager STORM 840.

Generation of C. neoformans Strains Carrying the Luciferase Gene under the Control of Wild-type or Mutated Forms of the APP1 Promoter—Plasmid pSK/APP1/NAT1/3′UTR was generated as follows: the NAT1 gene under the control of the C. neoformans actin (ACT) promoter was amplified from the pNAT1 vector (kindly provided by Dr. John Perfect, Duke University Medical Center, Durham, NC) using primers XB-NAT-F (5′-CTAATACTTAGAGCGAGATGCTGAGCTGAGATCGG-3′) and XB-NAT-R (5′-CGGCCCTAGAGAGAAGAGTGAAGAATACCTGCT-3′), which contain XbaI sites (bold and underlined). The resulting fragment was cloned into pCR2.1-TOPO vector (Invitrogen), generating pCR-NAT1. The LUC gene was amplified using primers Luc5′, 5′-GATCCCTCTCCGCTTTCTTTTCC-3′, and Luc3′, 5′-GATCCCTCTCCGCTTTCTTTTCC-3′, and plasmid pGKL3 basic vector (Promega) as a template. The resulting fragment was digested with HindIII and BamHI and cloned into the HindIII- and BamHI-digested pCR-NAT1 vector. The resulting plasmid (pCR-NAT1/LUC) was digested with HindIII and EcoRV, yielding a fragment containing LUC/NAT1 that was subcloned into HindIII- and EcoRV-digested pSK vector (Invitrogen). The resulting plasmid, pSK/LUC/NAT1, was digested with EcoRV and SacI to insert the EcoRV-blunted and SacI-restricted 3′-UTR region of the APP1 gene from the pΔapp1 plasmid (3), generating pSK/LUC/NAT1/3′UTR. Next, a 800-bp fragment corresponding to the APP1 promoter was obtained by digesting pΔapp1 plasmid with XhoI and EcoRI and subcloned into XhoI and SalI-blunted restricted pSK/LUC/NAT1/3′UTR plasmid creating plasmid pSK/APP1/LUC/NAT1/3′/UTR, which was biologically transformed into C. neoformans WT and GAL7:IPC1 to generate IPC1/APP1/LUC and GAL7:IPC1/APP1/LUC strains. This 800-bp APP1 sequence was chosen because it was highly predicted to be the promoter of APP1: 1) it represents the 5′-UTR sequence immediately upstream of the APP1 mRNA transcribed region (GenBank™ accession number AY965856); 2) it contains the TATA box (−72 bp from ATG) and two putative consensus sequences for transcription factors AP-2 (−236 bp from ATG) and ATF (−139 bp from ATG); and 3) it was highly predicted to be a promoter region when blasted into the Biomatics & Molecular Analysis Section (BIMAS) at the National Institutes of Health (bimas.dcrt.nih.gov/molbio/proscan/index.html).

Plasmid pSK/S′UTR/LUC/NAT1/3′UTR was generated as follows: a 876-bp 5′-UTR fragment corresponding to the upstream untranslated region of the APP1 promoter was amplified from genomic DNA using primers APP15-XhoI, 5′-GATCCCGCTCCGCTTTCTCTTTTCC-3′, and APP13-ClaI, 5′-GATCCCTCTCCGCTTTCTTTTCC-3′, which contain XhoI and ClaI sites, respectively (bold and underlined). The resulting fragment was subcloned into pCR2.1 TOPO vector, generating the pCR/S′UTR plasmid. This plasmid was digested with XhoI and HindIII and the resulting 876-bp fragment was subcloned into the XhoI- and HindIII-restricted pSK/LUC/NAT1/3′UTR plasmid. The resulting vector pSK/S′UTR/LUC/NAT1/3′UTR was transformed into the GAL7:IPC1 strain to generate the negative control GAL7:IPC1/S′UTR/LUC strain.

Plasmid pSK/S′UTR/ACT/LUC/NAT1/3′UTR was generated as follows: 5′-UTR of the APP1 locus was amplified from genomic DNA using primers APP15-XhoI, 5′-GATCCCGCTCCGCTTTCTTTTCC-3′, and APP13-ClaI, 5′-GATCCCTCTCCGCTTTCTTTTCC-3′, which contain XhoI and ClaI sites, respectively (bold and underlined). C. neoformans actin promoter (ACT) was amplified using primers Act5′, 5′-GATCCCGCTCCGCTTTCTTTTCC-3′, and Act3′, 5′-GATCCCTCTCCGCTTTCTTTTCC-3′, which contain Clal and HindIII sites, respectively (bold and underlined). These fragments were digested with the corresponding enzymes and cloned into the XhoI- and HindIII-restricted pSK/LUC/NAT1/3′UTR plasmid. The resulting plasmid, pSK/S′UTR/ACT/LUC/NAT1/3′UTR, was trans-

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**Table 1**

| No. | Strains                      | Ref. |
|-----|------------------------------|------|
| 1   | IPC1 (wild-type)             | 20   |
| 2   | GAL7:IPC1                   | 20   |
| 3   | IPC1/APP1/LUC               | This study |
| 4   | GAL7:IPC1/APP1/LUC          | This study |
| 5   | IPC1/ACT1/UTR/LUC           | This study |
| 6   | GAL7:IPC1/UTR/LUC           | This study |
| 7   | IPC1/366/LUC                | This study |
| 8   | GAL7:IPC1/S′UTR/LUC + Tel/366/LUC Tel/366/LUC | This study |
| 9   | GAL7:IPC1/S′UTR/LUC + Tel/Δap2/LUC Tel/Δap2/LUC | This study |
| 10  | GAL7:IPC1/S′UTR/LUC + Tel/Δatf/LUC Tel/Δatf/LUC | This study |
| 11  | GAL7:IPC1/S′UTR/LUC + Tel/Δatf + Δap2/LUC Tel/Δatf + Δap2/LUC | This study |
| 12  | GAL7:IPC1/S′UTR/LUC + Tel/map2/LUC Tel/map2/LUC | This study |
| 13  | GAL7:IPC1/S′UTR/LUC + Tel/map2/LUC Tel/map2/LUC | This study |
| 14  | IPC1/APP1/LUC/Δatf2          | This study |
| 15  | GAL7:IPC1/APP1/LUC/Δatf2     | This study |
formed into the C. neoformans WT H99 strain to generate the positive control IPC1/ACT-LUC strain.

Plasmid pSK/S' UTR/366/LUC/NAT1/3' UTR was generated as follows: pΔapp1/ADE plasmid (3) was digested with HindIII and EcoRI, yielding a 366-bp fragment corresponding to the end of the APP1 promoter. The 366-bp fragment was blunted and subcloned into HindIII-restricted and -blunted pSK/S' UTR/LUC/NAT1/3' UTR vector. The resulting construct, pSK'S' UTR/366/LUC/NAT1/3' UTR, was transformed into the C. neoformans WT H99 strain to generate the IPC1/366/LUC strain.

The above plasmids were sequenced before the biologic transformation to confirm appropriate insertion of the corresponding fragments.

Stable resistant transformants were selected for further analysis after five passages onto non-selectable YPD agar medium, and genomic DNA was extracted and subjected to Southern analysis with appropriate probes to identify a double crossover event at the APP1 locus without ectopic integrations (data not shown).

Plasmid pTel/366/LUC was generated as follows: the 366/LUC fragment was amplified from the pSK/APP1/LUC/NAT1/3' UTR plasmid using primers 366A, 5'-CACGATATCCAGTAAACTGATGTTAC-TGGAAAC-3', and 366B, 5'-CACGATATCCATTACACATTTTGTGAAGGTATAC-3', which contain EcoRV sites (bold and underlined). The resulting fragment was subcloned in pCR2.1 TOPO vector generating plasmid pCR-366/LUC, which was digested with EcoRV. The resulting fragment was subcloned into the KpnI-restricted and -blunted pTel/ACT-LUC plasmid (kindly provided by Dr. John Perfect, Duke University Medical Center, Durham, NC), generating the pTel/366/LUC plasmid.

Three deletions were generated by site-directed mutagenesis (Invitrogen) in the 366-bp fragment. The ATF consensus sequence (TGACGCTCA) was deleted in the pCR-366/LUC plasmid using primers ATPdel1, 5'-TACTATGATGCACCCTGCTTCAAAGTCGACT-3', and ATPdel2, 5'-TACGACGATGACTACATAGTATAGCTGTAAG-3', and the resulting Δatf/LUC fragment was cloned into pTel/ACT-HYG generating pTel/Δatf/LUC plasmid. The AP-2 consensus sequence (CCCCCGGGCC) was deleted in the pCR-366/LUC plasmid using primers AP2del1, 5'-CCCGCTACTAGGGACATGTGGCCT-TTGCTC-3', and AP2del2, 5'-ACATGCCCCATCATGGGGGTGTC-GCCGTTTT-3', and the resulting Δap2/LUC fragment was cloned into pTel/ACT-HYG generating the pTel/Δap2/LUC plasmid. The ATF consensus sequence was also deleted in the pTel/Δap2/LUC plasmid, resulting in the pTel/Δatf+Δap2/LUC plasmid. Additionally, the ATF consensus sequence TGACGCTCA was mutated into TGAAATCA by PCR site-directed mutagenesis using primers ATFmtu1, 5'-TGTAGT-CACCTGCTGATAAACAAATC-3', and ATFmtu2, 5'-ATGACAGGTGAACATGAGGATGAGGACATGAA-3', and the pCR-366/LUC plasmid as a template. The resulting fragment was subcloned into the pTel/ACT-HYG plasmid, generating pTel/matf/LUC plasmid. The AP-2 consensus sequence CCCCCCGGGCC was mutated into CCCCCGAC by PCR site-directed mutagenesis using primers AP2mut1, 5'-ATGAGGACATGTTCCCAGACTGCT-3', and AP2mut2, 5'-GGGGGAACTGCTCCAGAGGATGAGGACATGAA-3', and pCR-366/LUC plasmid as a template. The resulting fragment was subcloned into the pTel/ACT-HYG plasmid, generating pTel/map2/LUC plasmid. The above plasmids were sequenced prior to biologic transformation to make sure that the desired deletions and mutations had occurred.

These plasmids were transformed into the GAL7/JPC1/S' UTR/LUC strain according to Toffaletti et al. (18). The transformants were patched onto hygromycin (HYG) plates, then three times onto YPD, and finally onto HYG plates. Transformants that did not grow on the final HYG plates were processed from the YPD plates for genomic DNA extraction according to Casadavall and Perfect (19). Southern analysis of undigested DNA was performed to confirm epoampic integration (data not shown). From the above transformation reactions, transformant numbers 20, 8, 24, 6, 7, and 24 were chosen and designated C. neoformans Tel/366/LUC, Tel/Δatf/LUC, Tel/Δap2/LUC, Tel/Δatf+Δap2/LUC, Tel/mapf/LUC, and Tel/map2/LUC strains, respectively.

Ipc1 and Luciferase Enzymatic Activities—Ipc1 activity was performed as described previously (20). Luciferase activity was performed according to the Promega protocol described in the Luciferase Reporter Gene Assay. Proteins were extracted according to Luberto et al. (20). Then, 20 μl of cell lysate was added to 100 μl of luciferase assay reagent (Promega) and the production of luciferase was immediately measured by a Reporter Microplates Luminometer (Turner Designs). Results were normalized per 1 μg of proteins.

Lipid Treatments—The C. neoformans strains were grown on the appropriate medium in a shaking incubator for 24 h at 30°C. Cell pellets were washed twice with SDW, resuspended in fresh medium, and incubated in the appropriate medium in a shaking incubator for 16 h at 30°C. Next, cell pellets were washed twice with SDW, resuspended in SDW, and counted. Then, 5 × 10⁵ cells/ml were inoculated in 40 ml of appropriate medium containing 0, 5, 10, and 20 μM 1,2-dioctanoylglycerol (DiC8) for 2 h and 30 min at 30°C in a shaking incubator. Proteins were then extracted, quantified, and luciferase activity was measured.

Identification of Putative C. neoformans ATF2 Gene, Cloning, and Disruption—To identify the potential transcription factor(s) responsible for APP1 activation, we blasted the human AP-2 gene family, AP-2α (NP_003211), AP-2β (NP_003212), AP-2 β-like (NP_758430), AP-2 (CA121171), AP-2γ (NP_003213), and AP-2α (CA123520) and human ATF gene family, ATF1 (P18846), ATF2 (NM_001880), ATF3 (P18847), ATF4 (P18848), ATF5 (Q9Y2D1), ATF6 (P18850), and ATF7 (P17544) into the C. neoformans H99 Duke University Genome Data base (creo.genetics.duke.edu/blast.html). The search identified one sequence with an E value of 1e-11 corresponding to chr2-piece9 for the human Atf2 or Atf7 transcription factors, whereas we could not find any significant homology with any other ATF or AP-2 transcription factors. Thus, we focused our attention of the sequence identified in chr2-piece9, which was named putative C. neoformans ATF2. The sequence was retrieved, translated, and the amino acid sequence was aligned with human ATF2 and ATF7 genes. A putative open reading frame containing the basic region and the leucine zipper characteristic of the bZIP domain was identified. Thus, the 5' UTR fragment corresponding to the upstream region of the ATF2 gene was amplified using primers Atf51, 5'-CAATC-CTAGAATTTCCATCTTCTCCCCCTCAGG-3', and Atf52, 5'-CAGATCTTGAAGGAAAGGTTGTAAGG-3', which contain XbaI and BamHI sites, respectively (bold and underlined), and C. neoformans H99 genomic DNA as a template. Next, the 3' UTR fragment corresponding to the downstream region of the ATF2 gene was amplified using primers Atf31, 5'-CTCGAG-GGTATCGATAAGCTTTA-3', and Atf32, 5'-CTCGAG-CTCGAG-AAGGATGAGGCTGTTTT-3', and the resulting fragment was cloned into the pCR/LUC plasmid. The resulting fragment was subcloned into the pTel/366/LUC plasmid, generating pTel/S' UTR/LUC plasmid. These plasmids were transformed into the C. neoformans WT H99 strain to generate the positive control IPC1/ACT-LUC strain.
Ipc1-DAG Signaling Regulates APP1 Transcription

RESULTS AND DISCUSSION

Ipc1 Regulates Transcription of APP1—In previous studies, we found that APP1 mRNA levels are regulated by Ipc1 modulation (16). Thus, we investigated whether Ipc1 regulates APP1 gene expression at the transcriptional level. A nuclear run-on assay was used to measure the level of APP1 mRNA upon Ipc1 modulation. Fig. 2 shows that when Ipc1 is up-regulated (GAL7/IPC1 strain grown in galactose) the transcription of IPC1 and APP1 mRNA increases compared with the WT strain. These results suggest that up-regulation of Ipc1 increases APP1 transcription. The nuclear run-on assay was also performed in conditions in which Ipc1 was down-regulated (glucose). We found that, using this method, neither IPC1 nor APP1 mRNA transcripts decrease in the GAL7/IPC1 strain grown on glucose compared with the WT strain (data not shown), suggesting that the sensitivity of the method is not sufficient.

To investigate how the APP1 transcription is regulated by Ipc1, a ∼800-bp fragment upstream of the ATG of the APP1 gene was fused to the luciferase reporter gene of the firefly Photinus pyralis in both WT and GAL7/IPC1 strains, producing IPC1/APP1:LUC and GAL7/IPC1/APP1:LUC strains. The IPC1/APP1:LUC and GAL7/IPC1/APP1:LUC strains were grown in glucose and galactose, and luciferase activity was measured. Up-regulation of Ipc1 determined a significant increase of luciferase activity in the GAL7/IPC1/APP1:LUC strain, confirming previous results with the nuclear run-on assay, in which up-regulation of Ipc1 increases APP1 transcription. Importantly, there was no significant difference in the luciferase activity between glucose and galactose cultures of the IPC1/APP1:LUC strain, suggesting that the different carbon source does not affect APP1 transcription (Fig. 3A). Interestingly, under conditions in which Ipc1 is down-regulated (GAL7/IPC1/APP1:LUC grown on glucose), luciferase activity does not decrease below the wild-type level. These results suggest that when Ipc1 is down-regulated, potential compensatory mechanism(s) may exist leading to an increase of DAG level by pathways other than that regulated by Ipc1. This hypothesis is supported by evidence that a decrease of DAG under conditions of Ipc1 down-regulation is transitory and occurring in the very early log-phase of growth (14). It is also possible that because of the leakiness of the IPC1 promoter, the effect of GAL7 down-regulation cannot be measured using these assays at a given time point. On the other hand, in previous studies we showed that APP1 mRNA levels analyzed by reverse transcriptase-PCR are significantly decreased when Ipc1 is down-regulated (16). It is also possible that under conditions in which Ipc1 is down-regulated, the APP1 mRNA level decreases as a result of an increased degradation of APP1 mRNA. Clearly, this potential regulatory mechanism awaits further characterization.

Thus, to better address whether APP1 transcription can be turned “on,” “off,” and on again by Ipc1, the GAL7/IPC1/APP1:LUC strain was first grown on galactose, then switched to glucose, and finally placed back into galactose medium. Under these conditions, both Ipc1 and

FIGURE 2. Regulation of mRNA level of APP1 by Ipc1 in C. neoformans. The wild-type (IPC1) and the GAL7/IPC1 strains were grown in galactose. mRNAs were extracted, labeled with [32P]dUTP, and hybridized with ACT (white arrow), IPC1 (white arrowhead), and APP1 (black short arrow) cDNAs. A and B are autoradiograms, and C is the quantitative analysis of radioactive signals of A and B using a phosphorimager. The up-regulation of Ipc1 caused an increase in the mRNA levels of IPC1 and APP1 compared with the wild-type strain. Data are representative of three separate experiments.

FIGURE 3. Regulation of luciferase activity by Ipc1 in IPC1/APP1:LUC and in GAL7: IPC1/APP1:LUC strains. A, IPC1/APP1:LUC cells grown in glucose (GLU) showed similar luciferase activity of cells grown on galactose (GAL). Up-regulation of Ipc1 activity (GAL7: IPC1/APP1:LUC in galactose) caused an increase of luciferase activity, §, p < 0.001, GAL7: IPC1/APP1:LUC in GLU versus GAL7/IPC1/APP1:LUC in GAL. No statistical difference between IPC1/APP1:LUC in GLU versus IPC1/APP1:LUC in GAL. B, luciferase and Ipc1 activity of the GAL7/IPC1/APP1:LUC strain grown in galactose for 18 h, switched in glucose for 3 h, and then switched to galactose for 3 and 9 h. Modulation of Ipc1 activity regulates luciferase activity. Light intensity is 0.1 μg of protein. Data are representative of at least three independent experiments.
Ipc1-DAG Signaling Regulates APP1 Transcription

Luciferase activities were measured. As shown in Fig. 3, B and C, down-regulation of Ipc1 decreases luciferase activity, whereas switching of the cells to Ipc1-inducing conditions (galactose) induces an increase of luciferase activity. These results suggest that modulation of Ipc1 regulates APP1 transcription.

As a positive control, we fused the actin promoter to the luciferase gene into the C. neoformans H99 wild-type strain, creating a IPC1/ACT:LUC strain. As a negative control, we used a ~876-bp long nucleotide sequence (5′-UTR) upstream of the APP1 promoter and fused it to the luciferase gene in the GAL7:IPC1 strain, creating a GAL7:IPC1/5′UTR:LUC strain (see "Materials and Methods" for details). Strains were grown in glucose or galactose, and luciferase activity was measured. As expected, the GAL7:IPC1/5′UTR:LUC strain did not show any luciferase activity, under conditions in which Ipc1 was up- or down-regulated (Fig. 4). The IPC1/ACT:LUC strain showed a high level of luciferase activity that was not modulated by the different carbon sources (Fig. 4). Importantly, luciferase activity was detected at a high level, as expected, in IPC1/ACT:LUC. Taken together, these results suggest that the nucleotide sequence corresponding to the APP1 promoter activates luciferase activity and that APP1 transcription is under the control of Ipc1.

DAG Activates APP1 Transcription—Because in previous studies we showed that C. neoformans Ipc1 regulates the level of phytoceramide and DAG (14), we next wondered whether treatment with DAG or phytoceramide would affect luciferase activity. Thus, the IPC1/APP1-LUC, GAL7:IPC1/5′UTR:LUC, and IPC1/ACT:LUC strains were treated with different concentrations of DAG, and luciferase activity was measured. We found a dose- and time-dependent increase of luciferase activity in the IPC1/APP1:LUC strain treated with DAG (Fig. 5, A and B). Importantly, treatment with DAG did not increase luciferase activity in the IPC1/ACT:LUC strain (Fig. 5C), and did not activate luciferase in the GAL7:IPC1/5′UTR:LUC strain (Fig. 5D). Because Ipc1 also regulates the level of phytoceramide (14), we wondered whether luciferase activity would be modulated by treatment with ceramides or phytosphingosine. We found no effect on luciferase activity when cells were exposed to different concentrations of C2-, C6-ceramide, C6-phytytoceramide, or phytosphingosine (data not shown). These results suggest that DAG may be the effecter of the Ipc1 regulation over the APP1 promoter.

APP1 Transcription Is Regulated by ATF and AP-2 cis-Acting Elements—To identify potential sites of regulation of the APP1 promoter, a blast search for consensus sequences for transcription factors was performed (bimas.dcrt.nih.gov/molbio/proscan/index.html). The search revealed the presence of two putative consensus sequences normally recognized by transcription factors AP-2 and ATF, located at ~236 bp (AP-2) and ~139 bp (ATF) from ATG, respectively. In mammalian cells, it has been suggested that DAG may exert a regulatory effect on the transcription factor(s) ATF/cAMP-responsive element (21–23). Also, it has been proposed that AP-2 mediates transcriptional activation either in response to DAG and the DAG-PKC pathway or in response to the cAMP-PKA pathway (24). According to our previous findings in which Ipc1 activates C. neoformans Pck1 through DAG in vitro (14) and in vivo (15), it was particularly intriguing to hypothesize that Ipc1-DAG would regulate APP1 transcription through ATF or/and AP-2.

FIGURE 4. Fusion of ACT and 5′-UTR sequences with LUC in C. neoformans and analysis of luciferase activity. Luciferase activity of IPC1/ACT:LUC and GAL7:IPC1/5′UTR:LUC in glucose (GLU) and galactose (GAL). IPC1/ACT:LUC strain produces a high level of luciferase activity, which is not modulated by the different carbon source; no statistical difference between IPC1/ACT:LUC in GLU versus IPC1/ACT:LUC in GAL. The GAL7:IPC1/5′UTR:LUC strain did not show any detectable luciferase activity. Light int/sec/1 μg protein, light intensity/s/1 μg of protein. Luciferase experiments were performed at least three times.

FIGURE 5. Effect of DAG on luciferase activity. IPC1/APP1:LUC, GAL7:IPC1/5′UTR:LUC, and IPC1/ACT:LUC strains were grown in glucose and treated with DiC8. A, treatment with 5, 10, or 20 μM of DiC8 for 2 h and 30 min at 30 °C caused an increment of luciferase activity in the IPC1/APP1:LUC strain in a dose- and time-dependent manner: A, 5, 10, and 20 μM DiC8 does not have effect on luciferase activity in IPC1/ACT:LUC strain, or D, in the GAL7:IPC1/5′UTR:LUC strain. No statistical difference between IPC1/ACT:LUC treated with 20 versus 0 μM. Light int/sec/1 μg protein, light intensity/s/1 μg of protein. All experiments were performed at least three times.

FIGURE 6. Fusion of 366 bp of APP1 promoter with LUC and generation of mutated episomal strains in C. neoformans. Diagrams illustrating the IPC1/366LUC strain and the episomal plasmids used for transformation into the GAL7:IPC1/5′UTR:LUC strain. See "Materials and Methods" for details.
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To address this hypothesis, we focused on a stretch of 366 bp of the APP1 promoter containing the two cis-acting elements (AP-2 and ATF). The 366-bp fragment was fused to the LUC gene and it was either integrated into the APP1 locus in the WT strain yielding the IPC1/366:LUC strain (Fig. 6), or transformed episomally in the GAL7:IPC1/5’S'UTR:LUC strain using the pTel/366:LUC plasmid (Fig. 6) yielding the Tel/366:LUC strain. Fig. 7A shows that the 366-bp fragment is sufficient to activate luciferase activity in both strains in which the 366:LUC fragment is integrated chromosomally or episomally (Fig. 7A), and luciferase activity controlled by the 366-bp fragment is not significantly different from that one controlled by the ~800-bp APP1 promoter (Fig. 3A).

Importantly, up-regulation of Ipc1 significantly increased luciferase activity when the LUC gene is driven episomally by the 366-bp fragment (Fig. 7A), similar to what was observed in the GAL7:IPC1/APP1:LUC strain (Fig. 3A). Next, the IPC1/366:LUC and Tel/366:LUC strains were treated with DAG, and luciferase activity was measured. As shown in Fig. 7, B and C, following DAG treatment, luciferase activity increased in a dose-dependent manner. These results clearly suggest that the 366-bp fragment is sufficient to drive luciferase activation by Ipc1 and DAG in strains in which the 366-bp fragment is either integrated or episomal.

Next, we determined the effect of the deletion or mutations of AP-2 and ATF consensus sequences on luciferase activity. Thus, plasmid constructs carrying AP-2 or ATF or AP-2/ATF deletions or mutations were transformed episomally in the GAL7:IPC1/APP1:LUC strain (Fig. 8), as indicated. Stable transformants were screened by Southern analysis of undigested genomic DNA and transformants carrying only the episomal plasmid(s) were selected (data not shown). In the deletion mutant strains, the AP-2 or ATF consensus sequence were removed from the plasmid constructs, the AP-2 or ATF consensus sequences and ATF consensus sequence were removed from the plasmid constructs, whereas deletion of AP-2 dramatically increased luciferase activity by 20-fold (Fig. 8A). When the ATF consensus sequence was mutated (from TGACGTCA into TGAAATCA), the...
basal luciferase activity was not altered (Fig. 8A), although DAG activation was completely abrogated (Fig. 8B). Treatment with DAG did not increase luciferase activity in either the AP-2-deleted or mutated strains (Fig. 8B), suggesting that DAG might have a negative effect on AP-2 activity.

These results might suggest that the two CG nucleotides in the ATF consensus sequence are responsible for the DAG-mediated activation of APP1 transcription. This hypothesis is supported by studies in mammalian cells, in which mutation of these two nucleotides in the ATF cis-acting element abrogates the activation of the ecto-5'-nucleotidase
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Figure 10. A model for the activation of APP1 by the sphingolipid pathway and DAG in C. neoformans. Ipc1 regulates the level of DAG that activates APP1 transcription, potentially through an ATF- and AP-2-mediated mechanism.

With these studies, we propose that C. neoformans Atf2 is under the control of the Ipc1-DAG pathway. This control may be exerted by Pkc1 because C. neoformans Pkc1 is also regulated by the Ipc1-DAG pathway (14, 15). Importantly, preliminary studies showed that treatment with calphostin C, which, in addition to the mammalian PKC, also inhibits C. neoformans Pkc1 (14), abolishes the activation of luciferase by DAG in the IPC1/APP1:LUC strain, supporting the hypothesis that DAG may activate Atf2 through Pkc1. Considering that Ipc1 controls Pkc1 in C. neoformans (15) and that, in mammalian cells, Atf2 phosphorylation is required for its activation (31, 32), the hypothesis that Ipc1-DAG-Pkc1 may activate APP1 transcription through the Atf2 transcription factor and ATF and/or AP-2 cis-acting elements is particularly attractive.

Conclusions—It is anticipated that the study of regulation of gene transcription(s) that favors C. neoformans to escape the host response and cause disease is a future area of investigation given the recent completion of the genome sequence of this organism (33) and the construction of C. neoformans microarrays (genome.wustl.edu/projects/cneoformans/microarray/). With this study, we developed the use of luciferase as a reporter for gene expression as an important additional tool for studying gene transcription of C. neoformans in vitro. By using this approach, we dissected the Ipc1-App1 pathway and we propose that the biochemical mechanism by which Ipc1 regulates APP1 transcription is through the production of DAG and the potential activation of Atf2 (Fig. 10). The presence of stimulatory (ATF) and putative inhibitory (AP-2) factors in the regulation of APP1 transcription would suggest that the gene may be activated only under certain circumstances, perhaps during different stages of the cryptococcal infection. In previous studies, App1 protein was found in sera of AIDS patients affected by cryptococcosis and, given its characteristic nature of being anti-phagocytic, C. neoformans might regulate the production of App1 protein to escape internalization by phagocytic cells and down-regulate it once yeast cells would be internalized. This intriguing regulation would support the hypothesis that, to survive, pathogens would need to show adaptation and plasticity during their interaction with the host. In being both an extra- and intracellular microorganism, C. neoformans may have developed unique features, such as the production of App1 protein (or other antiphagocytic factors such as the polysaccharide capsule), which can be positively and negatively controlled and, thus, allow the fungus to counteract the immune response depending on the immune status of the host. How these microbial features are regulated and how they promote fungal survival within the host is a very attractive area of investigation.

4 M. Del Poeta, unpublished results.
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Lydia Mare, Roberta Iatta, Maria Teresa Montagna, Chiara Luberto and Maurizio Del Poeta

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