THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 43, pp. 36055–36064, October 28, 2005

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

Received for publication, July 5, 2005, and in revised form, August 4, 2005 Published, JBC Papers in Press, August 29, 2005, DOI 10.1074/jbc.M507285200

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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 for ATF and AP-2 transcription factors. Mutagenesis analysis of these sequences revealed that they play a key role in the regulation of *APP1* transcription: ATF is an activator, whereas AP-2 in a negative regulator. Finally, we identified a putative Atf2 transcription factor, which is required for *APP1* transcription and under the control of Ipc1-DAG pathway. These studies provide novel regulatory mechanisms of the sphingolipid pathway involved in the regulation of gene transcription of *C. neoformans*.

Inositol-phosphorylceramide synthase 1 (Ipc1)[3] is a fungal-specific enzyme of the sphingolipid pathway (Fig. 1) that regulates the level of phytoceramide and diacylglycerol (DAG), two well established bioactive molecules in mammalian cells, which regulate key cellular functions such as cell growth and viability (1–6). On the other hand, the role of these lipids in signaling in yeast cells is poorly understood.

Although the presence of sphingolipid enzymes has been demonstrated in *Saccharomyces cerevisiae* (7), and in pathogenic fungi, such as *Aspergillus fumigatus* (8), *Candida albicans*, and *Cryptococcus neoformans* (9), studies of sphingolipid-mediated signaling transduction in pathogenic fungi are in their infancy. Studies in *S. cerevisiae* showed that Ipc1 modulates the level of phytoceramide and DAG (10) but whether these lipids regulate signaling in this microorganism has yet to be elucidated. Whereas in mammalian cells DAG is a well known activator of protein kinase C (PKC), DAG does not activate the fungal homolog Pkc1 in *S. cerevisiae* (11, 12) or in *C. albicans* (13). Thus, if DAG regulates signaling in *S. cerevisiae* or *C. albicans* this regulation would be exerted through proteins other than Pkc1.

On the other hand, *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 (14). In recent studies, we showed that Ipc1 activates Pkc1 in *C. neoformans* through a DAG-dependent mechanism (14). This activation is mediated by the C1 domain of Pkc1 and regulates the localization and function of laccase (15), an enzyme that catalyzes melanin, which is required for the pathogenicity of *C. neoformans*.

Additional studies revealed that Ipc1 also plays a role in the regulation of phagocytosis of *C. neoformans*, through the modulation of a novel fungal factor called antiphagocytic protein 1 (App1), which inhibits the attachment and ingestion of fungal cells by macrophages (16). Ipc1 controls App1 at the mRNA level, suggesting a transcriptional or post-transcriptional activation. Therefore, we sought to examine the possibility that Ipc1 and the lipids that it modulates, such as phytoceramide or/and DAG, may regulate the transcriptional activation of fungal factor(s), such as App1.

To study the mechanism of the transcriptional regulation of the *APP1* gene by Ipc1, the *APP1* promoter was fused to the firefly luciferase gene in both *C. neoformans* wild-type and the *GAL7*:*IPC1* strain, in which Ipc1 can be regulated by growing yeast cells in glucose or galactose. We find that the luciferase activity is modulated by the expression of Ipc1. 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, whereas treatment with ceramide/phytoceramide or sphingosine/phytosphingosine does not affect *APP1* transcription. We identified two consensus sequences in the *APP1* promoter for AP-2 and ATF cis-acting elements. Deletion of the ATF consensus sequence in the *APP1* promoter abolishes luciferase activity, whereas mutation of ATF abrogates the DAG-dependent activation. Deletion or mutation of

[*] This work was supported in part by the Burroughs Wellcome Fund, National Institutes of Health Grant AI56168 (to M. D. P.), the Centers of Biomedical Research Excellence Program of the National Center for Research Resources Grant RR17677 Project 2 (to M. D. P.) and Project 6 (to C. L.), and National Science Foundation/Experimental Program to Stimulate Competitive Research (EPSCoR) Grant EPS-0132573 (to C. L.). 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.

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[3] The abbreviations used are: Ipc1, inositol-phosphorylceramide synthase 1; DAG, diacylglycerol; ATF, activating transcription factor; LUC, luciferase; NAT1, nourseothricin acetyltransferase 1; HYG, hygromycin B; ACT, actin; UTR, untranslated region; APP1, antiphagocytic protein 1; SDW, sterile-distilled water; PKA, protein kinase A; PKC, protein kinase C.

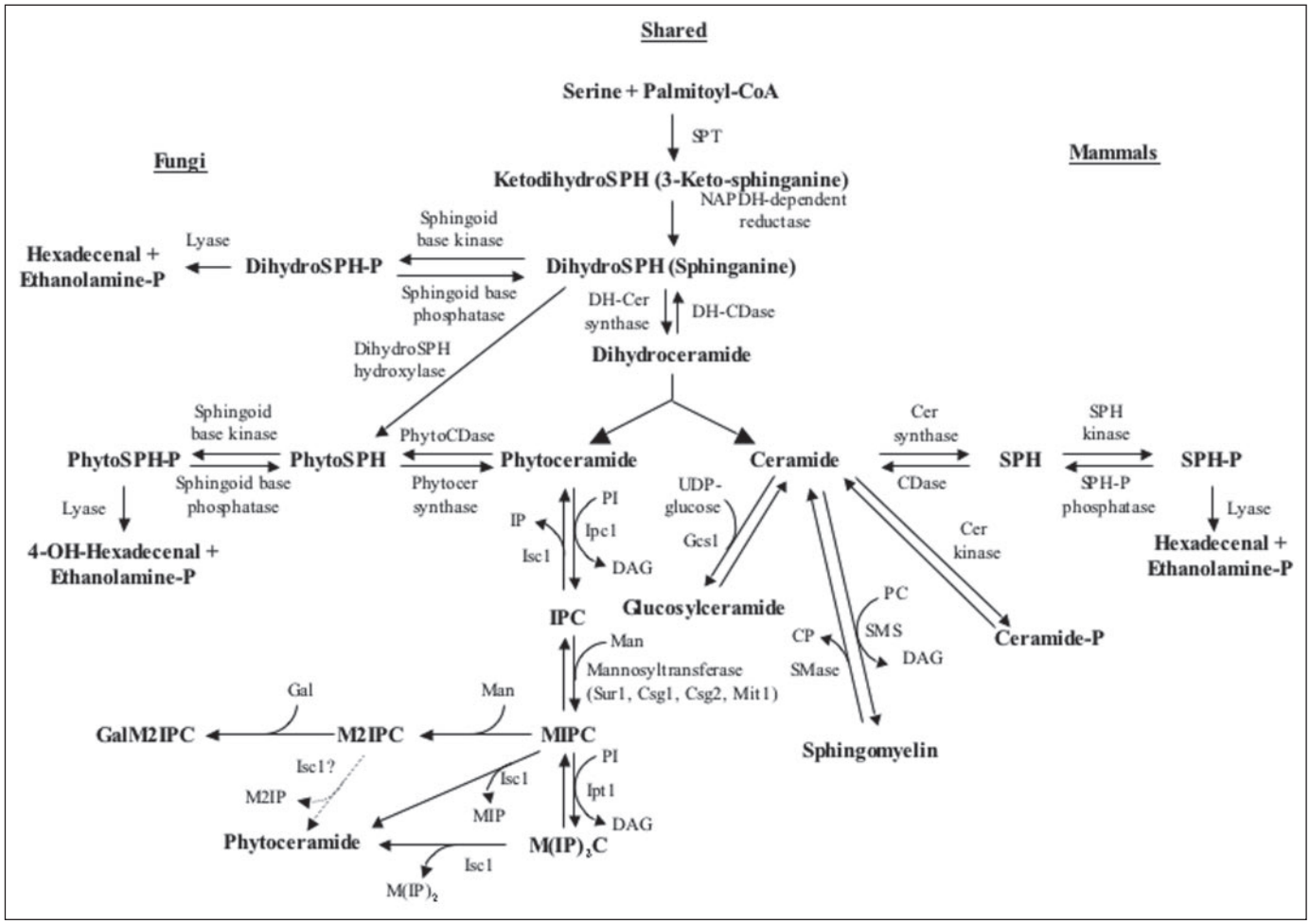

FIGURE 1. **The sphingolipid biosynthetic pathways**. A scheme of sphingolipids and their metabolizing enzymes in fungal and mammalian systems. *SPT*, serine palmitoyltransferase; *CDase*, ceramidase; *DH-Cer*, dihydroceramide; *DH-CDase*, dihydroceramidase; *Gcs1*, glucosylceramide synthase 1; *IPC*, inositol phosphorylceramide; *Ipt1*, inositol phosphotransferase 1; *Isc1*, inositol phosphosphingolipids-phospholipase C1; *MIPC*, mannose-inositol-P-ceramide; *M(IP)$_2$C*, mannose-(inositol-P)$_2$-ceramide; *M2IPC*, dimannose-inositol-P-ceramide; GalM2IPC, galactose-dimannose-inositol-P-ceramide; *IP*, inositol phosphate; *CP*, choline phosphate; *PI*, phosphatidylinositol; *PC*, phosphatidylcholine; SMase, sphingomyelinase; *SMS*, sphingomyelin synthase; *SPH*, sphingosine. Adapted from Ref. 6.

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*/*ACT*:*LUC*, *GAL7*:*IPC1*/*5′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*/Δ*atf2* and *GAL7*:*IPC1*/*APP1*:*LUC*/Δ*atf2* 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 *GAL7IIPC1* 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 β-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 × 10^7 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 nM NaCl, 5 mM $MgCl_2$). 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 $MgCl_2$, 1 mM $MnCl_2$, 2 mM dithiothreitol, 0.5 mM ATP, GTP, CTP, 100 μCi of [α-^{32}P]UTP, and 1 units of RNasin). The

TABLE ONE

List of *C. neoformans* strains used in this study

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/ACT:LUC*	This study
6	*GAL7:IPC1/5′UTR:LUC*	This study
7	IPC1/366:LUC	This study
8	*GAL7:IPC1/5′UTR:LUC* + pTel/*366:LUC* (Tel/*366:LUC*)	This study
9	*GAL7:IPC1/5′UTR:LUC* + pTel/Δ*ap2:LUC* (Tel/Δ*ap2:LUC*)	This study
10	*GAL7:IPC1/5′UTR:LUC* + pTel/Δ*atf:LUC* (Tel/Δ*atf:LUC*)	This study
11	*GAL7:IPC1/5′UTR:LUC* + pTel/Δ*atf*+Δ*ap2:LUC* (Tel/Δ*atf*+Δ*ap2:LUC*)	This study
12	*GAL7:IPC1/5′UTR:LUC* + pTel/*map2:LUC* (Tel/*map2:LUC*)	This study
13	*GAL7:IPC1/5′UTR:LUC* + pTel/*matf:LUC* (Tel/*matf:LUC*)	This study
14	*IPC1/APP1:LUC*/Δ*atf2*	This study
15	*GAL7:IPC1/APP1:LUC*/Δ*atf2*	This study

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 nytran membranes in which actin, *IPC1*, and *APP1* cDNA were previously immobilized using a slot blot apparatus. Before adding the labeled mRNA, the nytran 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 nytran 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 phosphoscreen 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*/*LUC*/*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′-CTAA**TCTAGA**GCGAGGATGTGAGCTGGAGAGCGG-3′) and XB-NAT-R (5′-CGCG**TCTAGA**GAAGAGATGTAGAAACTAGCTTCC-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 Luc3′, 5′-GATCTTTCCGCCCTTCTT-3′, and Luc5′, 5′-GCATGCCAGAGATCCTAT-3′, and plasmid pGL3 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 EcoRI-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 that 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 biolistically 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 BioInformatics & Molecular Analysis Section (BIMAS) at the National Institutes of Health (bimas.dcrt.nih.gov/molbio/proscan/index.html).

Plasmid pSK/5′*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′-CAT**CTCGAG**TGAGTACTGGATCTG-3′, and APP13-HindIII, 5′-GAA**AAGCTT**TCATTGCTTAACGGTATTG-3′, which contain XhoI and HindIII sites, respectively (bold and underlined). The resulting fragment was subcloned into pCR2.1 TOPO vector, generating the pCR/5′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/5′*UTR*/*LUC*/*NAT1*/3′*UTR* was transformed into the *GAL7:IPC1* strain to generate the negative control *GAL7:IPC1*/5′*UTR:LUC* strain.

Plasmid pSK/5′*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′-CAT**CTCGAG**TGAGTACTGGATCTG-3′, and APP13-ClaI, 5′-GAA**ATCGAT**TCATTGCTTAACGGTATTG-3′, which contain XhoI and ClaI sites, respectively (bold and underlined). *C. neoformans* actin promoter (*ACT*) was amplified using primers Act5, 5′-CAA**ATCGAT**GCTGCGAGGATGTGA-3′, and Act3, 5′-GTT**AAGCTT**TTGGCGGAGTTTACTAAT-3′, which contain ClaI 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/5′*UTR*/*ACT*/*LUC*/*NAT1*/3′*UTR*, was trans-

formed into the *C. neoformans* WT H99 strain to generate the positive control *IPC1*/*ACT*:*LUC* strain.

Plasmid pSK/*5′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/*5′UTR*/*LUC*/*NAT1*/*3′UTR* vector. The resulting construct, pSK*5′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 biolistic 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′-CAC**GATATC**CAGTAAACTGTAGTTTAC-TGGAAC-3′, and 366B, 5′-CAC**GATATC**TTTACCACATTTGTA-GAGGTTTTAC-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 2,366-bp fragment was subcloned into the KpnI-restricted and -blunted pTel/*ACT*:*HYG* 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 (TGACGTCA) was deleted in the pCR-*366*:*LUC* plasmid using primers ATFdel1, 5′-TACTATGTAGTCACCTGTCAAAAGTCGTACT-3′, and ATFdel2, 5′-TGACAGGTGACTACATAGTAATCGCTGTA-AG-3′, and the resulting Δ*atf*:*LUC* fragment was cloned into pTel/*ACT*:*HYG* generating pTel/Δ*atf*:*LUC* plasmid. The AP-2 consensus sequence (CCCCGCGGC) was deleted in the pCR-*366*:*LUC* plasmid using primers AP2del1, 5′-CCCCCACTATGGGGACATGTTCGCC-TTGTCC-3′, and AP2del2, 5′-ACATGTCCCCATAGTGGGGGTC-GCCGATTTT-3′, and the resulting Δ*ap2*:*LUC* fragment was cloned into pTel/ACT:*HYG* generating the pTel/Δ*ap2*:*LUC* plasmid. The ATF consensus sequence was also deleted in the pTel/Δ*ap2*:*LUC* plasmid, resulting in the pTel/Δ*atf*+Δ*ap2*:*LUC* plasmid. Additionally, the ATF consensus sequence TGA**CG**TCA was mutated into TGA**AA**TCA by PCR site-directed mutagenesis using primers ATFmut1, 5′-TGTAGT-CACCTGTCATGAAATCAAAAG TCGT-3′, and ATFmut2, 5′-TC-ATGACAGGTGACTACATAGTAATCGCT-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 CCCCGC**GG**C was mutated into CCC-CGC**AA**C by PCR site-directed mutagenesis using primers AP2mut1, 5′-ATGGGGACATGTCCCCGCAACTCGCCTTGTC-3′, and AP2mut2, 5′-GCGGGGACATGTCCCCATAGTGGGGGTCG-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 biolistic transformation to make sure that the desired deletions and mutations have occurred.

These plasmids were transformed into the *GAL7*:*IPC1*/*5′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 Casadevall and Perfect (19). Southern analysis of undigested DNA was performed to confirm episomal 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/Δ*atf*:*LUC*, Tel/Δ*ap2*:*LUC*, Tel/Δ*atf*+Δ*ap2*:*LUC*, Tel/*matf*:*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 2 times 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 YP medium, and counted. Next, 5×10^6 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_758438), AP-2Δ (CAI21171), AP-2γ (NP_003213), and AP-2ε (CAI23520) 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 (cneo.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′-CAA**T-CTAGA**TTTCATCACTTCTCCCCTCTCCGC-3′, and Atf52, 5′-CA-A**GGATCC**TGAGTGATGAAAGAGGTGGTAAAG-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′-CAA**CTCGAG**TTGGTCATG-GTGTGATCATTCTTC-3′, and Atf32, 5′-CAA**GGTACC**AAGAGA-AGGGAGATTAGATCG-3′, which contain XhoI and KpnI sites, respectively (bold and underlined), and the *C. neoformans* H99 genomic DNA as a template. Finally, the *ACT*:*HYG* was amplified from the pTel/*ACT*:*HYG* plasmid using primers Hyg/Act1, 5′-CAA**GGATCC**-TGCGAGGATGTGAGCTGGAGAGCG-3′, and Hyg/Act2, 5′-CAA-C**CTCGAG**GTCGACGGTATCGATAAGCTTTA-3′, which contain BamHI and XhoI sites, respectively (bold and underlined). The *5′UTR*, *ACT*: *HYG*, and *3′-UTR* fragments were digested with XbaI + BamHI,

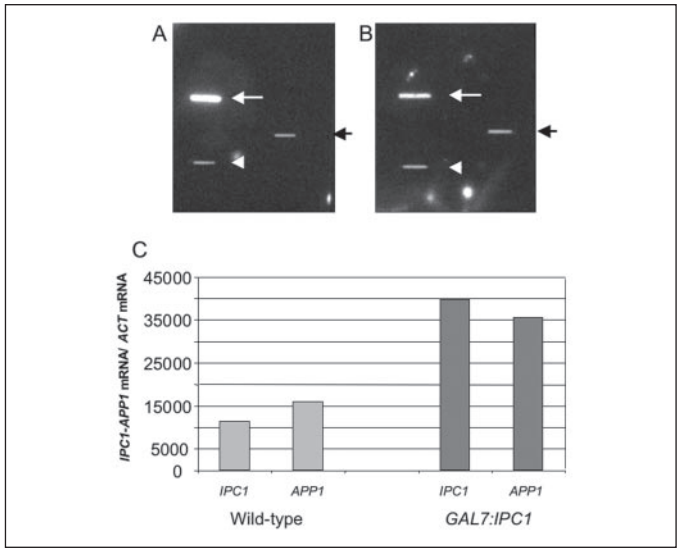

FIGURE 2. **Regulation of mRNA level of *APP1* by Ipc1 in *C. neoformans*.** The wild-type (*IPC1*) and the *GAL7*:*IPC1* strains were grown in galactose. mRNAs were extracted, labeled with [^{32}P]dUTP, and hybridized with *ACT* (*white long arrow*), *IPC1* (*white arrowhead*), and *APP1* (*black short 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.

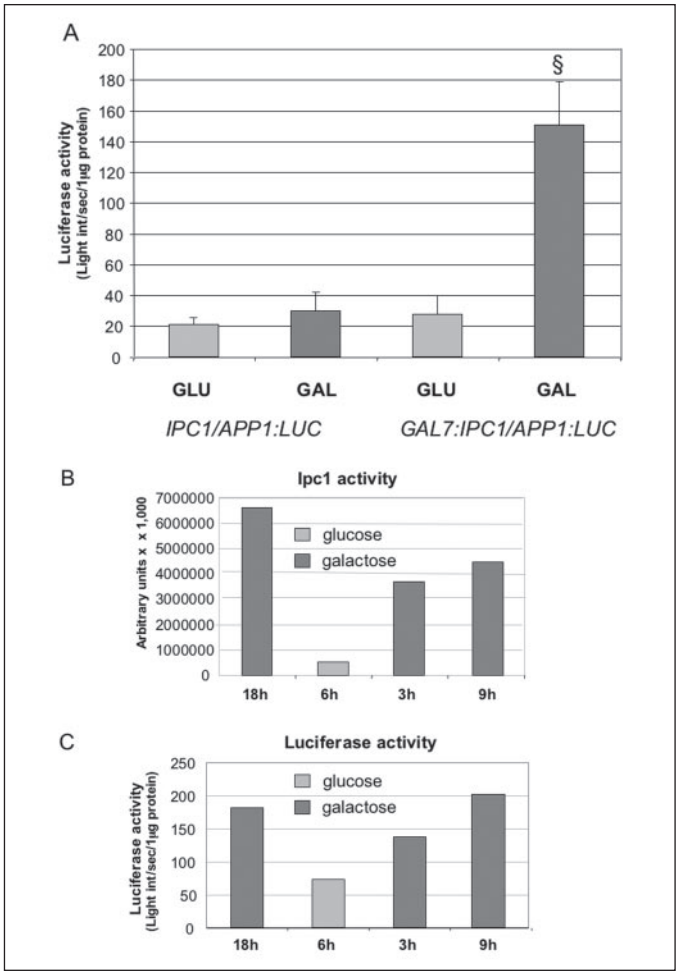

FIGURE 3. **Regulation of luciferase activity by Ipc1 in *IPC1/APP1:LUC* and in *GAL7*:*IPC1/APP1:LUC* strains.** *A*, *IPC1/APP1:LUC* cells grown in glucose (GLU) showed similar luciferase activity of cells grown on galactose (GAL). Up-regulation of Ipc1 activity (*GAL7*:*IPC1/APP1:LUC* in galactose) 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 int/sec/1 µg of protein*, light intensity/s/1 µg of protein. Data are representative of at least three independent experiments.

BamHI + XhoI, and XhoI + KpnI, respectively, and cloned into XbaI- and KpnI-restricted pSK vector, yielding the pSK/*5′UTR*/*HYG*/*3′UTR* plasmid, which was biolistically transformed into the *C. neoformans* WT H99 strain and *GAL7*:*IPC1* strain producing *IPC1*/*APP1*:*LUC*/Δ*atf2* and *GAL7*:*IPC1*/*APP1*:*LUC*/Δ*atf2* strains.

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

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. 3*A*). 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 *GAL7* 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). Thus, it is 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

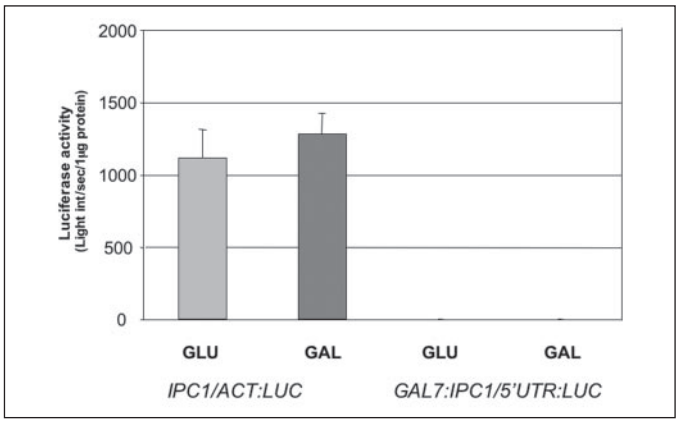

FIGURE 4. **Fusion of *ACT* and 5′-*UTR* sequences with *LUC* in *C. neoformans* and analysis of luciferase activity.** Luciferase activity of *IPC1/ACT:LUC* and *GAL7:IPC1/5′UTR:LUC* in glucose (*GLU*) and galactose (*GAL*). *IPC1/ACT:LUC* strain produces a high level of luciferase activity, which is 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 μ*g protein*, light intensity/s/1 μg of protein. Luciferase experiments were performed at least three times.

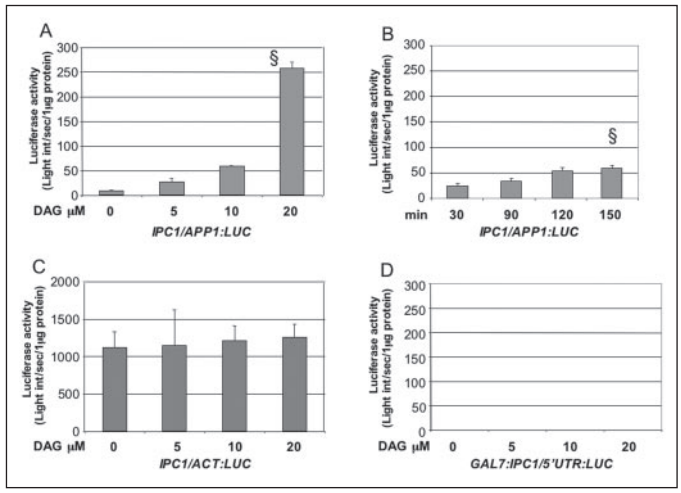

FIGURE 5. **Effect of DAG on luciferase activity.** *IPC1/APP1:LUC*, *GAL7:IPC1/5′UTR:LUC*, and *IPC1/ACTIN:LUC* strains were grown in glucose and treated with DiC8. *A*, treatment with 5, 10, or 20 μM of DiC8 for 2 h and 30 min at 30 °C caused an increment of luciferase activity in the *IPC1/APP1:LUC* strain in a dose- (*A*) and time (*B*)-dependent manner; *A*, §, $p < 0.001$, *IPC1/APP1:LUC* treated with 10 *versus* 20 μM; *B*, §, $p < 0.05$, *IPC1/APP1:LUC* treated for 150 min *versus* for 30 min. *C*, treatment with 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.

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. 5*C*), and did not activate luciferase in the *GAL7:IPC1/5′UTR*:LUC strain (Fig. 5*D*). 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-phytoceramide, or phytosphingosine (data not shown). These results suggest that DAG may be the effector of the Ipc1 regulation over the *APP1* promoter.

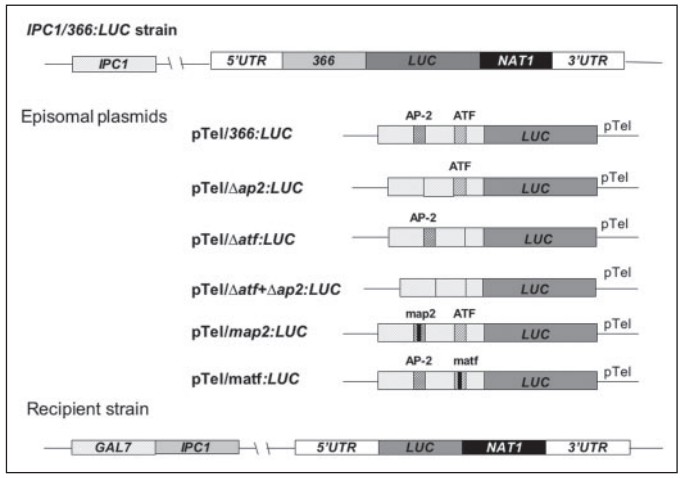

FIGURE 6. **Fusion of 366 bp of *APP1* promoter with *LUC* and generation of mutated episomal strains in *C. neoformans*.** Diagrams illustrating the *IPC1/366:LUC* strain and the episomal plasmids used for transformation into the *GAL7:IPC1/5′UTR:LUC* strain. See "Materials and Methods" for details.

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* Pkc1 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.

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′UTR:LUC* strain using the pTel*/366:LUC* plasmid (Fig. 6) yielding the Tel/*366:LUC* strain. Fig. 7*A* 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. 7*A*), and luciferase activity controlled by the 366-bp fragment is not significantly different from that one controlled by the ~800-bp *APP1* promoter (Fig. 3*A*). Importantly, up-regulation of Ipc1 significantly increased luciferase activity when the *LUC* gene is driven episomally by the 366-bp fragment (Fig. 7*A*), similar to what was observed in the *GAL7:IPC1/APP1:LUC* strain (Fig. 3*A*). 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:IPC1/5′UTR: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 *APP1* promoter, whereas in the mutated strains two nucleotides in the ATF or AP-2 consensus sequence were mutated (see "Materials and Methods" for details). As shown in the figure, deletion of ATF abolished luciferase activity, whereas deletion of AP-2 dramatically increased luciferase activity by ~20-fold (Fig. 8*A*). When the ATF consensus sequence was mutated (from TGACGTCA into TGAAATCA), the

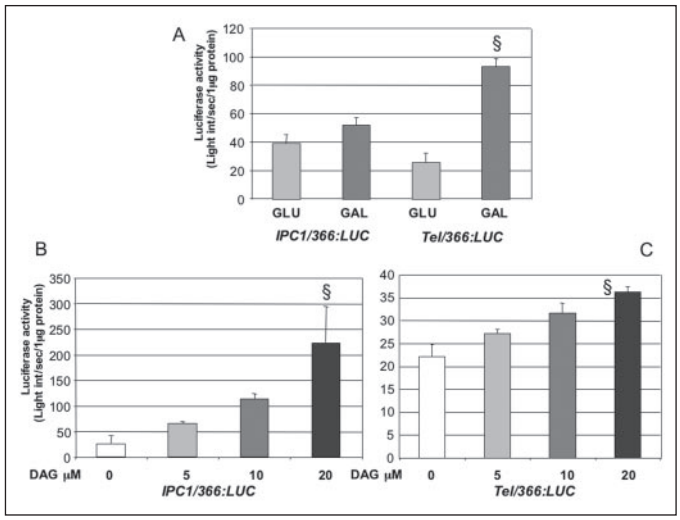

FIGURE 7. **Luciferase activity of *IPC1/366:LUC* and Tel/*366:LUC* strains grown in glucose and galactose, and upon DAG treatment.** *A*, *IPC1/366:LUC* strain grown in glucose (*GLU*) or galactose (*GAL*) showed similar luciferase activity; no statistical difference between *IPC1/366:LUC* in GLU *versus* GAL. The Tel/*366:LUC* strain in galactose (*GAL*) showed a significant increase of luciferase activity compared with glucose (*GLU*); §, $p <$ 0.001, Tel/*366:LUC* in GLU *versus* GAL. *B*, effect of DiC8 on luciferase activity in the *IPC1/366:LUC* strain grown in glucose. Luciferase activity significantly increases in a dose-dependent manner; §, $p < 0.01$, *IPC1/366:LUC* strain treated with 20 M *versus* 5 µM. *C*, effect of DiC8 on luciferase activity in the Tel/*366:LUC* strain grown in glucose. The treatment caused a significant increment of luciferase activity in a dose-dependent manner; §, $p <$ 0.05, 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.

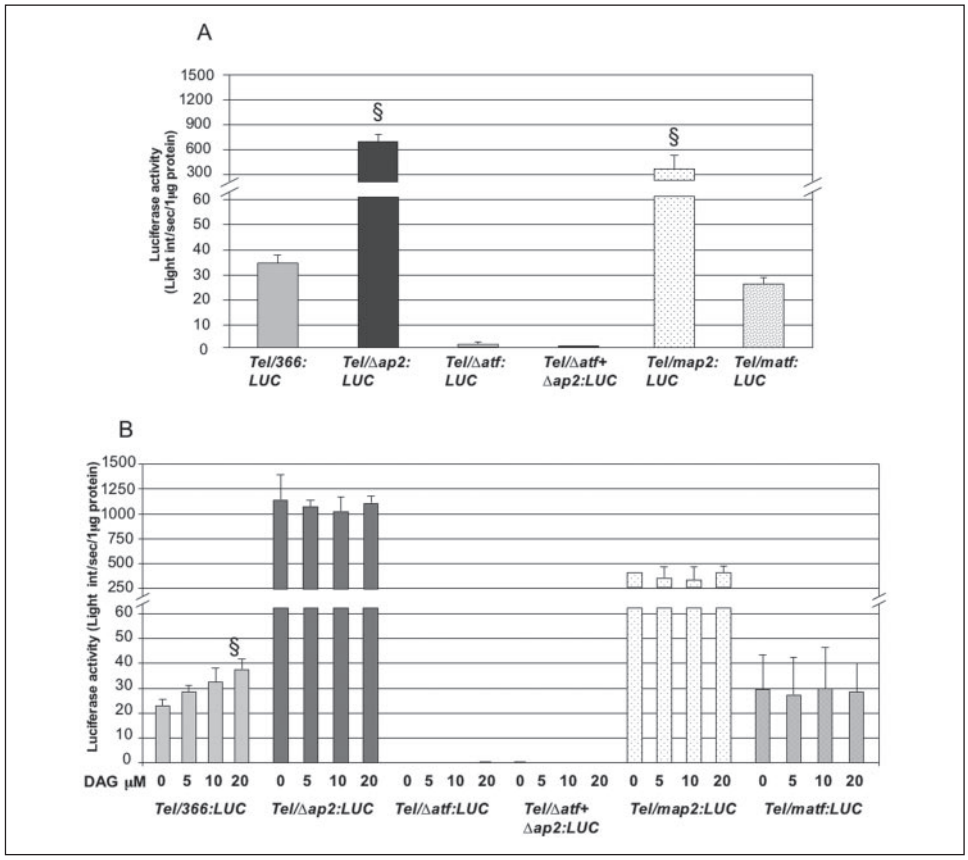

FIGURE 8. **Luciferase activity of mutated episomal strains grown in glucose and upon DAG treatment.** *A*, deletion of the AP-2 binding site, Tel/Δ*ap2:LUC* strain, caused a very strong increase of luciferase activity; §, $p < 0.0001$, Tel/Δ*ap2:LUC versus* Tel/*366:LUC*. Deletion of the ATF consensus sequence, Tel/Δ*atf:LUC* strain, or double deletion of ATF and AP-2, Tel/Δ*atf*+Δ*ap2:LUC*, caused a loss of luciferase activity. The mutation of two nucleotides in the AP-2 consensus sequence, Tel/*map2:LUC*, also causes a significant increased of the luciferase activity; §, $p < 0.0001$, Tel/*map2:LUC versus* Tel/*366:LUC*. Mutation of two nucleotides in the ATF consensus sequence, Tel/*matf:LUC*, did not significantly alter luciferase activity compared with that produced by the Tel/*366:LUC* strain. *B*, treatment with DAG does cause a dose-dependent increase of luciferase activity in the Tel/*366:LUC* strain (§, $p < 0.05$, 20 *versus* 0 µM), whereas treatment with DAG does not cause any increment of luciferase activity in ATF- or AP-2-deleted or mutated strains; no statistical difference between treatment 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.

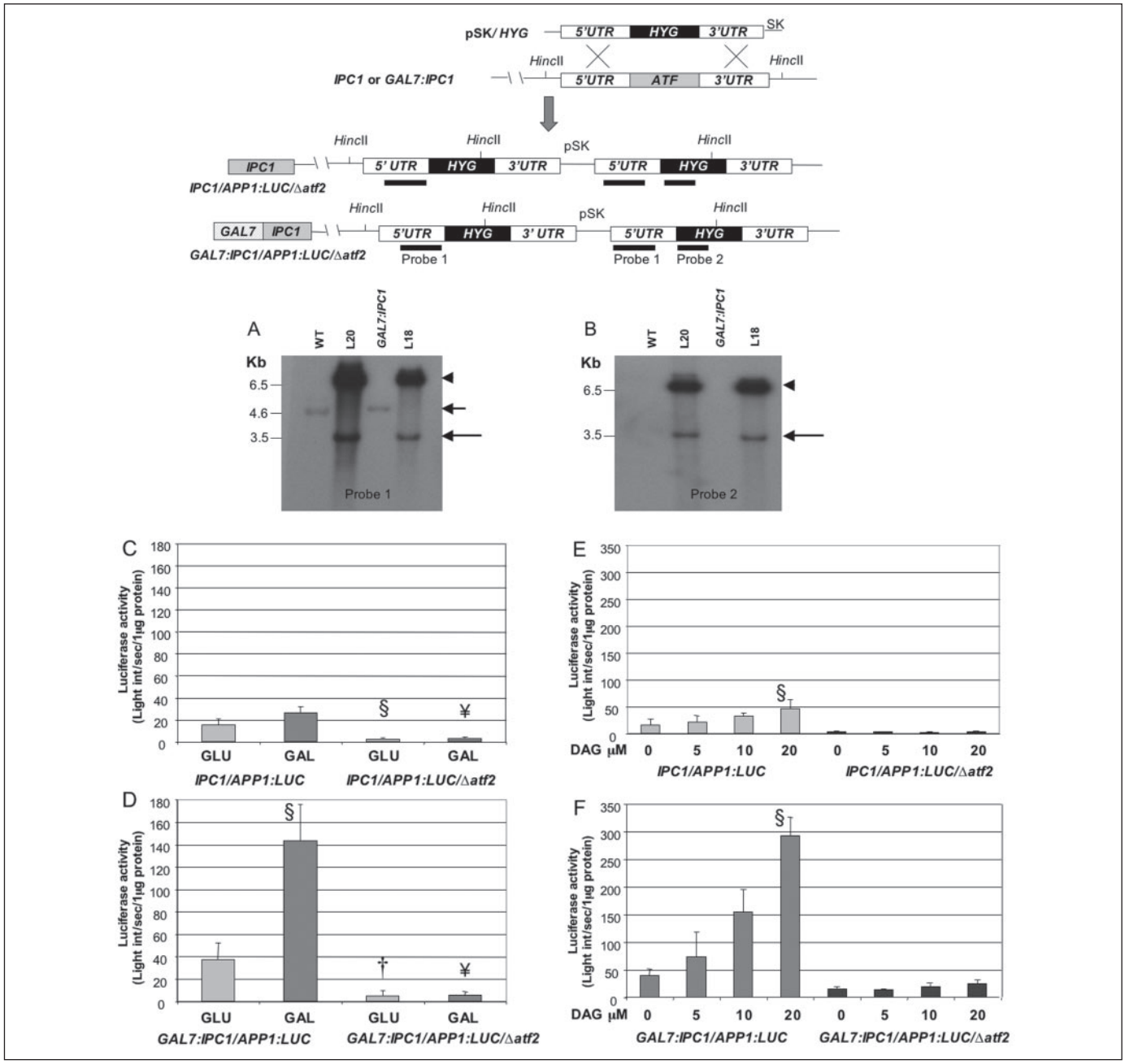

FIGURE 9. **Replacement of the *ATF* gene by *HYG* in *IPC1/APP1:LUC* and *GAL7:IPC1/APP1:LUC* strains.** A diagram illustrating the creation of the *IPC1/APP1:LUC/Δatf2* and *GAL7:IPC1/APP1:LUC/Δatf2* strains. *A*, Southern analysis using 5′-UTR probe (probe 1); and *B*, Southern analysis using hygromycin probe (probe 2) of genomic DNA extracted from wild-type parental strains (WT or *GAL7:IPC1*) and transformants L20 and L18 and digested with HincII. Transformants L20 and L18 showed a double crossover event (*long arrows*) with insert of plasmid loop (*arrowhead*). The *short arrow* represents the wild-type *ATF2* gene. *C*, luciferase activity of *IPC1/APP1:LUC* and *IPC1/APP1:LUC/Δatf2* strains in glucose (*GLU*) and galactose (*GAL*). Deletion of the *ATF2* gene caused a dramatic reduction of activity in both GLU and GAL; §, $p < 0.01$, *IPC1/APP1:LUC* in GLU *versus* *IPC1/APP1:LUC/Δatf2* in GLU; [yen], $p < 0.01$, *IPC1/APP1:LUC* in GAL *versus* *IPC1/APP1:LUC/Δatf2* in GAL. No statistical significant difference was found in luciferase activity of *IPC1/APP1:LUC* in GLU *versus* GAL. *D*, luciferase activity of *GAL7:IPC1/APP1:LUC* and *GAL7:IPC1/APP1:LUC/Δatf2* strains in glucose (*GLU*) and galactose (*GAL*). The significant increase of luciferase activity because of up-regulation of Ipc1 (§, $p < 0.001$, *GAL7:IPC1/APP1:LUC* strain in GAL *versus* GLU) is abrogated by the deletion of *ATF2* gene ([yen], $p < 0.001$, *GAL7:IPC1/APP1:LUC* strain in GAL *versus* *GAL7:IPC1/APP1:LUC/Δatf2* in GAL; †, $p < 0.01$, *GAL7:IPC1/APP1:LUC* strain in GLU *versus* *GAL7:IPC1/APP1:LUC/Δatf2* in GLU). *E*, DAG treatment of *IPC1/APP1:LUC* and *IPC1/APP1:LUC/Δatf2* strains with DiC8 grown in glucose. DAG treatment does not increase luciferase activity when the *ATF2* gene is deleted (no statistical difference between the *IPC1/APP1:LUC/Δatf2* strain treated with 20 *versus* 10 μM), whereas DAG activates luciferase activity in *IPC1/APP1:LUC* (§, $p < 0.05$, *IPC1/APP1:LUC* strain treated with 20 *versus* 0 μM). *F*, DAG treatment of *GAL7:IPC1/APP1:LUC* and *GAL7:IPC1/APP1:LUC/Δatf2* strains with DiC8 grown in glucose. *ATF2* deletion abolishes the luciferase activation by DAG treatment; §, $p < 0.01$, *GAL7:IPC1/APP1:LUC* strain treated with 20 *versus* 10 μM; no statistical difference between the *GAL7:IPC1/APP1:LUC/Δatf2* strain treated with 20 *versus* 0 μM. *Light int/sec/1 μg protein*, light intensity/s/1 μg of protein. All luciferase experiments were performed at least three times.

basal luciferase activity was not altered (Fig. 8*A*), although DAG activation was completely abrogated (Fig. 8*B*). Treatment with DAG did not increase luciferase activity in either the AP-2-deleted or mutated strains (Fig. 8*B*), 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

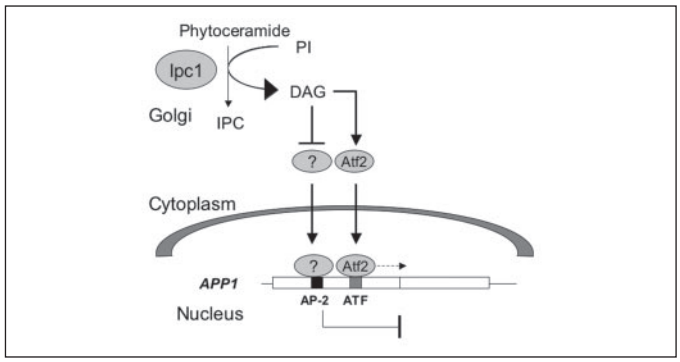

FIGURE 10. **A model for the activation of *APP1* by the sphingolipid pathway and DAG in *C. neoformans*.** Ipc1 regulates the level of DAG that activates *APP1* transcription, potentially through an ATF- and AP-2-mediated mechanism.

promoter by phorbol 12-myristate 13-acetate (25). Thus, our findings support other reports that describe the ATF cis-acting site as a phorbol 12-myristate 13-acetate/DAG responsive element (22, 23, 25).

Interestingly, because activation by DAG was also abolished when the AP-2 consensus sequence was either deleted or mutated, it would suggest that DAG might also have an effect on AP-2. Thus, we hypothesize that ATF is involved in the constitutive expression of *APP1* and that DAG positively controls *APP1* expression through ATF and negatively through AP-2. It is possible, however, that because luciferase activity is greatly increased upon AP-2 deletion or mutation, the addition of DAG cannot further activate luciferase or that DAG inhibits AP-2 activity that is lost when AP-2 is deleted or mutated. Further investigations are required to determine whether DAG and Ipc1 modulation would affect the binding of transcription factor(s) to ATF or/and AP-2 cis-acting elements of the *C. neoformans APP1* promoter.

Role of ATF2 Gene in APP1 Activation—Once the putative *C. neoformans ATF2* gene ORF was identified (see "Materials and Methods" for details), the putative *C. neoformans ATF2* gene was deleted in both *IPC1*/*APP1*:*LUC* and *GAL7*:*IPC1*/*APP1*:*LUC* strains, creating *IPC1*/*APP1*:*LUC*/Δ*atf2* and *GAL7*:*IPC1*/*APP1*:*LUC*/Δ*atf2* (Fig. 9, *A* and *B*). Strains were grown in glucose or galactose or treated with DAG, and luciferase activity was measured. We found that both strains show a dramatic reduction of luciferase activity, which is no longer under the control of Ipc1 modulation (Fig. 9, *C* and *D*) or DAG treatment (Fig. 9, *E* and *F*). Taken together, these results suggest that the *C. neoformans* Atf2 transcription factor may be activated by the Ipc1-DAG pathway to increase the transcription of *APP1* through ATF and/or AP-2 cis-acting elements of the *APP1* promoter.

The molecular interaction between Atf2 and the *APP1* promoter has yet to be elucidated. It is tempting to speculate that because an AP-2 homolog was not found in the *C. neoformans* genome data base the Atf2 transcription factor may bind to both ATF and AP-2 cis-acting elements and that the presence of DAG (perhaps promoting Atf2 phosphorylation) may favor the release of the binding to AP-2 while maintaining the binding to ATF. As a result, *APP1* transcription increases. On the other hand, it is possible that DAG negatively regulates a transcription factor yet to be identified that binds to the putative AP-2 consensus sequence. The model proposed in Fig. 10 takes into consideration this possibility. Clearly, this model awaits further biochemical characterization.

The question on the potential mechanism by which Ipc1-DAG activates the transcription factor(s) involved in the regulation of *APP1* transcription is also intriguing. In mammalian cells, although Atf2 (also known as CRE-BP-1) can be phosphorylated by PKC (26), ATF transcription factors, are mainly activated by the cAMP-PKA pathway (reviewed in Ref. 27). On the other hand, in the fission yeast *Schizosaccharomyces pombe*, the Atf1 transcription factor is homologous to the mammalian Atf2 and is positively regulated by stress-activated mitogen-activated protein kinase pathway but not by the cAMP-PKA pathway (28–30). When the *S. pombe* Atf1 protein (GenBank BAA09841) was blasted into the *C. neoformans* H99 data base, the same (and only) sequence identified by blasting the human Atf2 (Chr2-piece9) was found with an *E* value of 3e-22. This suggests that the putative *C. neoformans ATF2* gene that we identified is closely related to the human Atf2 and *S. pombe* Atf1 transcription factors. Importantly, the role and mechanisms by which ATF transcription factor(s) is regulated in human pathogenic fungi, such as *C. albicans*, *C. neoformans*, and *A. fumigatus*, are basically unexplored.

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,[4] 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.

Acknowledgments—We thank Dr. Besim Ogretmen and all members in his laboratory for helping with the luciferase assay. We also thank Drs. Yang, Yusuf Hannun, and Lina Obeid for helpful discussion, Daniel Taraskiewicz for technical assistance, and Dr. Jennifer Schnellmann for editorial assistance. We give special thanks to Dr. John Perfect for the gift of the NAT1 and HYG plasmids.

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

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J. Biol. Chem. 2005, 280:36055-36064.
doi: 10.1074/jbc.M507285200 originally published online August 29, 2005
