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*Cryptococcus neoformans* is a fungal pathogen that, after inhalation, can disseminate to the brain. Host alveolar macrophages (AMs) represent the first defense against the fungus. Once phagocytosed by AMs, fungal cells are killed by a concerted mechanism, involving the host-cellular response. If the cellular response is impaired, phagocytosis of the fungus may be detrimental for the host, since *C. neoformans* can grow within macrophages. Here, we identified a novel cryptococcal gene encoding antiphagocytic protein 1 (App1). App1 is a cryptococcal cytoplasmic protein that is secreted extracellularly and found in the serum of infected patients. App1 does not affect melanin production, capsule formation, or growth of *C. neoformans*. Treatment with recombinant App1 inhibited phagocytosis of fungal cells through a complement-mediated mechanism, and Δapp1 mutant is readily phagocytosed by AMs. Interestingly, the Δapp1 mutant strain showed a decreased virulence in mice deficient for complement C5 (A/Jcr), but it was hypervirulent in mice deficient for T and NK cells (Tge26). This study identifies App1 as a novel regulator of virulence for *C. neoformans*, and it highlights that internalization of fungal cells by AMs increases the dissemination of *C. neoformans* when the host cellular response is impaired.

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Identification of App1 as a regulator of phagocytosis and virulence of Cryptococcus neoformans

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Cryptococcus neoformans is a fungal pathogen that, after inhalation, can disseminate to the brain. Host alveolar macrophages (AMs) represent the first defense against the fungus. Once phagocytosed by AMs, fungal cells are killed by a concerted mechanism, involving the host-cellular response. If the cellular response is impaired, phagocytosis of the fungus may be detrimental for the host, since C. neoformans can grow within macrophages. Here, we identified a novel cryptococcal gene encoding antiphagocytic protein 1 (App1). App1 is a cryptococcal cytoplasmic protein that is secreted extracellularly and found in the serum of infected patients. App1 does not affect melanin production, capsule formation, or growth of C. neoformans. Treatment with recombinant App1 inhibited phagocytosis of fungal cells through a complement-mediated mechanism, and ∆app1 mutant is readily phagocytosed by AMs. Interestingly, the ∆app1 mutant strain showed a decreased virulence in mice deficient for complement C5 (A/Jcr), but it was hypervirulent in mice deficient for T and NK cells (Tge26). This study identifies App1 as a novel regulator of virulence for C. neoformans, and it highlights that internalization of fungal cells by AMs increases the dissemination of C. neoformans when the host cellular response is impaired.


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Nonstandard abbreviations used: alveolar macrophages (AMs); antiphagocytic protein 1 (App1); inositol phosphoryl ceramide synthase 1 (Ipc1); yeast nitrogen base (YNB); yeast peptone dextrose (YPD); yeast extract peptone (YP); bronchoalveolar lavage (BAL); 5% nonfat milk/1× PBS/0.1% Tween 20 (5% NFM/PBST); 5′ untranslated region (5′-UTR); open reading frame (ORF); 3′ untranslated region (3′-UTR); periodic acid–Schiff (PAS); differential display RT-PCR (DD-RT-PCR); inhibitory protein 1 (IP1); diacylglycerol (DAG).
macrophages (AMs) represent one of the cell types that inhibit and kill C. neoformans (12, 13). During the initiation process, T cells are activated, and through the release of macrophage-activating cytokines they promote the formation of granulomas, resulting in the destruction of the intracellular fungus or containment in a latent state (dormancy) (11). In individuals with impaired cellular immunity, C. neoformans grows intracellularly, eventually lysing the macrophage (reactivation-dissemination). The released organism can then infect other phagocytes, increasing the intracellular growth of the fungus (dissemination-proliferation). On the other hand, C. neoformans can also grow extracellularly, eluding phagocytosis through the production of specific fungal factor(s) that disable the recognition and response of the host immune system. Which component of the cryptococcal population, intracellular or extracellular, most affects the progression of the disease is still unclear (14–18). Here, we have identified a novel virulence factor of C. neoformans regulated by Ipcl that specifically inhibits the phagocytosis of yeast cells by AMs. Because of its distinctive function, we named this fungal factor antiphagocytic protein 1 (App1). We characterized the Ipcl-App1 pathway in the regulation of phagocytosis of C. neoformans by AMs using a combination of genetic and pharmacological approaches. We found that App1 inhibits attachment and ingestion of yeast cells by AMs through a complement-mediated mechanism. Interestingly, by testing Ipcl and App1 mutant strains in C5 and T-NK immunodeficient mouse models, we found that whereas Ipcl is required for the dissemination of C. neoformans from the lung to the brain in both animal models, lack of App1 favors the dissemination of yeast cells within macrophages when the T and NK cellular immunity is altered.

Methods

Strain and growing media. The following strains were used in this study: the Cryptococcus neoformans variety grubii serotype A strain H99 (WT), the M001 strain, an ade2 isogenic derivative of H99, GAL7:IPC1 strains 10 and 13 (created from M001) (9), two independent Δapp1 knockout strains (6 and 31, also created from M001), the IPC1Rec strain (created from GAL7:IPC1 10), and the Δapp1Rec strain (created from Δapp1 31). A synthetic medium, containing 6.7 g/l yeast nitrogen base (YNB) without amino acids, 1.3 g/l amino acid mix lacking adenine, 180 g/l sorbitol, 20 g/l glucose, and 20 g/l agar, was used for selecting the Δapp1 6 and 31 strains obtained from biolistic transformation. C. neoformans H99, M001, GAL7:IPC1, and Δapp1 strains were routinely grown on yeast peptone dextrose (YPD) medium. Yeast extract peptone dextrose (YPD) supplemented with 20 g/l glucose or 20 g/l galactose was used for down- and upregulation of IPC1 expression, respectively. Yeast extracts plated with 2% glucose and 200 U/ml hygromycin B (Calbiochem, San Diego, California, USA) were used to select and screen both IPC1Rec and Δapp1Rec strains. Antibody-coated erythrocytes (IgM/IgG) are from Advance Research Technologies (San Diego, California, USA). Complement/antibody-coated erythrocytes were created by incubating the IgM/IgG-coated erythrocytes with C5-deficient human serum (a gift from Stephen Tomlinson, Medical University of South Carolina, Charleston, South Carolina, USA) for 1 hour at 37°C. Sera of patients with AIDS and normal subjects were a gift from Angela Restrepo (Medellin, Colombia).

Phagocytosis assay. C. neoformans WT H99 and GAL7:IPC1 (10 and 13) strains were grown in YPD for 16 hours at 30°C in a shaker incubator at 250 rpm. Cells were washed twice with sterile PBS (pH 7.4), resuspended, and diluted into 10 ml of fresh YP broth with either glucose or galactose to a final density of 10⁸ cells per milliliter and incubated for 24 hours at 30°C in a shaker incubator at 250 rpm. C. neoformans Δapp1 mutant 6 and 31 strains were grown in YP with glucose for 24 hours at 30°C in a shaker incubator at 250 rpm. Cells were washed twice with PBS and resuspended in DMEM (glucose or galactose) containing 10% fresh mouse serum. Next, 6 × 10⁶ opsonized yeast cells were added to AMs, and plates were incubated at 37°C (5% CO₂) for 2 hours. IgM/IgG-coated or iC3b-IgM/IgG–coated erythrocytes were diluted in DMEM, and 6 × 10⁶ cells were added to AMs. Nonattached yeast or erythrocyte cells were then removed by washing with PBS. Plates were placed under an inverted microscope, and the phagocytic index was defined as the
percentage of yeast cells attached or ingested per number of macrophages per field, as previously described (9). For each experiment, at least four fields were observed in three different wells per strain or treatment condition. Results are expressed as geometric means ± SDs of the phagocytic indexes obtained for each experiment. Statistical analysis was performed with Student’s t test. A P value of less than 0.05 was considered to be significant. For ex vivo phagocytosis, mice were anesthetized with an intraperitoneal injection of 60 μl of xylazine/ketamine mixture, containing 95 mg of ketamine per kilogram of body weight and 5 mg of xylazine per kilogram of body weight. Then, C. neoformans WT, GAL7:IPC1, Δapp1, Δapp1Rec, and Δapp1Rec strains were inoculated intranasally. Three mice were used for each yeast strain. After 2 hours, mice were euthanized by CO2 inhalation, and AMs and yeast cells were collected by BAL. Cells were then centrifuged at 1,200 rpm and resuspended in 500 μl of PBS, and 10 μl was plated in a standard hemocytometer for the determination of the number of yeast cells attached and/or ingested by AMs (phagocytic index). Results represent the geometric means ± SDs of the phagocytic indexes obtained from three animals per strain.

**Differential display RT-PCR**. Differential display was performed according to the instructions for the GenHunter RNA-Image Kit (GenHunter Corp., Nashville, Tennessee, USA). Total RNA, obtained from C. neoformans WT H99 and GAL7:IPC1 strain 10 grown on glucose or galactose, was divided into three equal aliquots, and first-strand synthesis was performed using oligo-dT anchored with an A, C, or G. This cDNA was then subjected to PCR amplification with an arbitrary primer (AP) and the corresponding oligo-dT-A, -dT-C, or -dT-G, respectively. Four arbitrary primers were used (AP1, AP2, AP3, and AP4) for each oligo-dT, producing 12 different amplifications of gene transcription patterns for each strain (WT-glucose, GAL7:IPC1-glucose, WT-galactose, and GAL7:IPC1-galactose). For radioactive labeling, α-[32P]dATP was added in each PCR reaction. PCR products were separated at 1000 V in a 6% polyacrylamide gel. The gel was then transferred onto a 3M paper, dried, marked in each corner with radioactive ink, and yeast cells were collected by BAL. Cells were then centrifuged at 1,200 rpm and resuspended in 500 μl of PBS, and 10 μl was plated in a standard hemocytometer for the determination of the number of yeast cells attached and/or ingested by AMs (phagocytic index). Results represent the geometric means ± SDs of the phagocytic indexes obtained from three animals per strain.

**Ipcl activity, RT-PCR, and Western blot**. Ipcl activity was performed as previously described (9). RT-PCR directed against the APP1 gene was performed using 1 μg of total RNA for each group (WT-glucose, GAL7:IPC1-glucose, WT-galactose, and GAL7:IPC1-galactose) following the protocol described by the SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technology). First-strand synthesis of APP1 gene was made with primer IP13 (5'-AAT CAT CAA TGT TCG CAG CTC TTC-3') and second-strand synthesis of APP1 gene was made with primers IP13 and IP15 (5'-ATG ATG TCT TCC GCC ACT GCT GTA CAG-3'), yielding a 543-bp fragment. Actin-specific primers AC-2 (5'-AGT GAA GGT AGA CAA AGA GCC-3') and AC-1 (5'-CGC TAT CCT CCG TAT CGA TCT TGC-3') were used for the actin gene as a control, yielding a 543-bp fragment.

For Western blot, proteins from 24-hour cultures of WT-glucose, GAL7:IPC1-glucose, WT-galactose, and GAL7:IPC1-galactose were extracted as previously described (9), electrophoresed in 10% SDS-PAGE, and transferred onto a nitrocellulose membrane. Membrane was then blocked with 5% nonfat milk/1× PBS/0.1% Tween 20 (5% NFM/PBST). The primary antibody was anti-Ipcl at a 1:1,000 dilution in 5%NFM/PBST. The rabbit secondary antibody was anti-IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, California, USA) in 5%NFM/PBST. Membrane was washed generously in 5%NFM/PBST, treated with ECL (Amersham Pharmacia Biotechnology, Piscataway, New Jersey, USA), and exposed to Kodak X-Omat film. Analysis of Ipcl activity and APP1 mRNA and protein levels was performed in two independent GAL7:IPC1 transformants (nos. 10 and 13; see Figure 2).

**Disruption of the APP1 gene.** A disruption construct was made (see Figure 3) by using a series of molecular manipulations involving the APP1 gene and the 3.0-kb ADE2 marker genomic DNA fragment obtained from serotype A, strain H99. First, fragment A (784 bp) was generated by PCR using H99 genomic DNA as a template and primers IP(XhoI) (5'-GTT CTGAGGTTGGGATGATGGC GCTC ACTTTTTGG-3') and IP(EcoI) (5'-CTATGGAATT TCGATTATTGTGATGGGCTTAC-3'), containing a XhoI and EcoRI site, respectively (bolded and underlined). The 784-bp fragment contains sequence corresponding to the 5' upstream untranslated region (5'-UTR) of the APP1 open reading frame (ORF) (see Figure 3). This fragment was then digested with XhoI and EcoRI, producing fragment B (775 bp). Second, fragment C (888 bp) was generated using H99 genomic DNA as a template and primers IP(XbaI) (5'-CAA TCTA GAC ATG GCTATAG TGC CCGGCTACTTTTGG-3') and IP(EcoI) (5'-CTATGGAATT TCGATTATTGTGATGGGCTTAC-3'), containing an XbaI and EcoRI site, respectively (bolded and underlined). The 888-bp fragment contains sequence corresponding to the 5' upstream untranslated region (5'-UTR) of the APP1 open reading frame (ORF) (see Figure 3). This fragment was then digested with XhoI and EcoRI, producing fragment D (879 bp), which contains sequence homologous to the 3' downstream untranslated region (3'-UTR) of APP1 ORF (see Figure 3). Third, fragment B (5'-UTR) was combined with fragment D (3'-UTR), ligated, and cloned into the XhoI and...
XbaI sites of the SK+ pBluescript vector, generating plasmid pΔapp1. The pΔapp1 plasmid was sequenced to make sure that no mutations were introduced by PCR manipulations. Finally, the EcoRI-restricted ADE2 fragment was inserted into the EcoRI site located between 5′ UTR and 3′-UTR fragments to form the pΔapp1/ADE2 disruption construct. The pΔapp1/ADE2 was transformed into C. neoformans strain M001, using biolistic delivery of DNA, following the protocol as described by Toffaletti et al. (21). Transformants were grown on YNB-glucose without adenine (see above). Forty stable transformants were chosen, and genomic DNA was extracted and assayed for PCR and/or Southern blot analysis according to Sambrook et al. (22). Transformants showing integration of the pΔapp1/ADE2 construct at the APP1 locus were chosen and designed Δapp1 strains.

Reconstitution of the IPC1 and APP1 genes in the GAL7:IPC1 and Δapp1 mutant strains. The IPC1 gene expression in the GAL7:IPC1 strains is under the control of the GAL7 promoter, and its activity is modulated by glucose or galactose. To reconstitute the expression of IPC1, the GAL7 promoter was replaced with the IPC1 promoter using a plasmid cassette containing the upstream UTR of the IPC1 locus, the ADE2 gene, the hygromycin B gene, the IPC1 promoter, and the 5′-truncated IPC1 gene. This plasmid cassette was introduced into the GAL7:IPC1 strain 10. Hygromycin B-resistant transformants were selected and screened for homologous recombination at the IPC1 locus. Transformant 5 was selected and showed that Ipc1 activity is no longer modulated by glucose or galactose and is similar to the WT strain (data not shown). This transformant was named the IPC1Rec strain. To reconstitute the Δapp1 mutant strain, the WT APP1 gene was reintroduced into the Δapp1 mutant strain 31 by transforming the APP1 promoter fused to the APP1 gene linked to a HYG gene conferring hygromycin B resistance (23). Genomic DNA was isolated from six hygromycin B-resistant transformants and analyzed by Southern blot. Transformant 4 showed that both the Δapp1/ADE2 mutant allele and the WT APP1 gene were now present in the APP1 locus (see Figure 3) and that App1 protein was now produced (see Figure 3). This transformant was named the Δapp1Rec strain.

Production of App1 recombinant protein. App1 recombinant proteins were produced from C. neoformans serotype A using the corresponding APP1 cDNA. The pAPP1 cDNA plasmid from serotype A strain H99 was used as template for PCR with primers Dan5 (5′-CATGACCCACTGCTGAACTT-3′) and Dan3 (5′-CATGAAATTCGAAATCAATGT-3′), containing Xhol and EcoRI sites, respectively (bolded and underlined). The resulting fragment was then digested with Xhol and EcoRI and cloned into Xhol-EcoRI-restricted pBAD-HIS-B vector (Invitrogen, Life Technology), generating pBAD-His-App1. This vector contains App1 cDNA in frame with Xpress and HIS tags at the 5′ region of App1 gene. The pBAD-His-App1 vector was sequenced to ensure that no mutations were inserted by genetic manipulations and then transformed into E. coli TOP10 strain (Invitrogen, Life Technology). Induction of App1 proteins was obtained following the protocol described by the Invitrogen expression system. After an overnight culture on Luria Bertani (LB) medium containing 100 µg/ml ampicillin, induction of App1 was obtained by growing E. coli cells for an additional 4 hours at 37°C in 80 ml LB medium containing 50 µg/ml ampicillin and 2% arabinose in a shaker incubator at 250 rpm. Cells were then collected by centrifugation at 6,000 rpm and resuspended in lysis buffer (20 mM NaPO4 [pH 7.4], 20% glycerol, 1 mM PMSF, and 1 mg/ml lysozyme). After 30 minutes of incubation on ice, cells were lysed by sonication (six cycles of 1 minute each, 20% power). Cell lysate was then centrifuged at 20,000 g for 30 minutes at 4°C, and supernatant was further diluted with 20 mM sodium phosphate (pH 7.8, buffer A) and applied to a HiTrap chelating HP column (Amersham) (0.2 ml per minute) previously charged with 0.1 M NiSO4 according to the manufacturer’s instructions and equilibrated with buffer A. The column was then washed with five column volumes of buffer A, and bound proteins were eluted at 1 ml per minute with an increasing linear gradient of 20 mM sodium phosphate containing 0.5 M imidazole (pH 7.8, buffer B). Fractions were analyzed by Western blotting, and those with the highest amount of App1 (usually at 0.1–0.2 M imidazole) were combined (fractions 36–42), the buffer exchanged with 20 mM Tris-HCl (pH 8), 1 mM EDTA, 0.2 mM PMSF, and 0.005% Triton X-100 (buffer C) using PD-10 columns (Amersham) and applied to a HiTrap Q previously equilibrated with buffer C. After the column was washed with five column volumes of buffer C, bound proteins were eluted with an increasing linear gradient of buffer C containing 1.5 M NaCl, and fraction Q54 — with the highest ratio of App1 to contaminating proteins — was selected and used for cellular treatments. The lysate from cells expressing pBAD-His (mock) vector was used as a control and subjected to a purification protocol identical to the one used with pBAD-His-App1.

Virulence studies in murine models of cryptococcal meningitis. Four- to six-week-old male Tgε26 mice (Edward Balish, Medical University of South Carolina, Charleston, South Carolina, USA), and four- to six-week-old female A/Jcr mice (NCI/Frederick Laboratories) were used. The Tgε26 mice lack functional T and NK cells, whereas macrophages, monocytes, and granulocytes are present and functional (19, 24). The A/Jcr model from NCI/Frederick Laboratories has a slight C5 deficiency (25), and, as a consequence, they mount a predominant Th2 response (26–28). Mice were anesthetized with an intraperitoneal injection of 60 µl of xylazine/ketamine mixture, containing 95 mg of ketamine per kilogram of body weight and 5 mg of xylazine per kilogram of body weight. Wild-type H99 and the isogenic GAL7:IPC1, Δapp1, IPC1Rec, and Δapp1Rec strains were prepared by growing yeast cells for 24 hours at 30°C in YPD medium. The cells were pelleted, washed twice, and resuspended in PBS (pH 7.4) at a concentration of 2.5 × 10⁶ cells per milliliter. Ten A/Jcr and 14 Tgε26 mice were
infected with 5 × 10^4 cells of the H99, GAL7:IPC1, ∆app1, IPC1Rec, and ∆app1Rec strains in a volume of 20 µl through nasal inhalation. The mice were fed ad libitum and followed with twice-daily inspections. Mice that appeared moribund or in pain were sacrificed using CO₂ inhalation. Survival data from the mice experiment were analyzed using the Kruskal-Wallis test. A P value of less than 0.05 was considered to be significant.

Tissue-burden studies in murine models of cryptococcosis. Fifteen mice of each model were infected with 5 × 10^4 cells each of WT, GAL7:IPC1, ∆app1, IPC1Rec, and ∆app1Rec strains in a volume of 20 µl through nasal inhalation. C. neoformans strains were prepared as for the virulence studies. The mice were fed ad libitum and followed with twice-daily inspections. At selected time points after inoculation, five mice for each strain were sacrificed with CO₂ inhalation, and lungs and brains were removed, weighed, and homogenized in 10 ml sterile PBS using Stomacher 80 (Lab System, Fisher Scientific, Pittsburgh, Pennsylvania, USA) for 120 seconds at high speed. Serial dilutions were then plated onto YPD plates in triplicate. Plates were incubated at 30°C for 72 hours, yeast colonies were counted, and the number was recorded as the geometric mean(s) of the logarithm of CFU per organ. Statistical analysis was performed using Student’s t test, and a P value of less than 0.05 was considered to be significant. We also must note that two independent mutants for Ipc1 (GAL7:IPC1 10 and 13) and App1 (∆app1 6 and 31) were used for both survival and tissue-burden cultures studies. No significant differences were found between GAL7:IPC1 10 and 13 strains and between ∆app1 31 and 6 strains (data not shown).

Histology studies in murine models of cryptococcosis. Two mice of each model were infected with 5 × 10^4 cells each of WT, GAL7:IPC1, ∆app1, IPC1Rec, and ∆app1Rec strains in a volume of 20 µl through nasal inhalation. C. neoformans strains were prepared as for the virulence and tissue-burden studies. At selected time points (day 5 and 15 after infection for A/Jcr mice and day 6 and 13 after infection for Tg26 mice), lungs and brains were removed, placed in 10% formalin, and then embedded in paraffin. Sections were stained with periodic acid–Schiff (PAS) to identify fungal cell wall, with mucicarmine as an additional staining for the identification of C. neoformans capsule (data not shown), with hematoxylin and eosin to examine the host inflammatory response, and with anti-CD68 antibody to identify macrophages. Sections were examined by light microscopy.

Results
Ipc1 regulates phagocytosis of C. neoformans by alveolar macrophages. In our previous studies, we found that once C. neoformans is internalized by the macrophage-like cell J774.16, its intracellular growth is regulated by Ipc1 expression (9). Here, we investigated whether the level of Ipc1 expression would affect the phagocytosis of C. neoformans by mouse AMs. Phagocytosis (attached, ingested, and attached plus ingested) was increased by 2.4-fold when Ipc1 was downregulated (GAL7:IPC1 grown on glucose, P = 0.005) and decreased by 2.5-fold when Ipc1 was upregulated (GAL7:IPC1 grown on galactose, P = 0.004). Similar results were obtained with a second and independent GAL7:IPC1 transformant 13 (data not shown). No difference was observed between WT cells grown on glucose or galactose (Figure 1). Additionally, by testing the IPC1Rec strain, we found no significant difference in attached and/or ingested yeast cells with the WT strain (data not shown).

Ipc1 regulates the expression of App1. In order to determine the mechanism(s) by which Ipc1 regulates phagocytosis, we sought for downstream effector(s) of Ipc1 by differential display RT-PCR (DD-RT-PCR). C. neoformans WT H99 and GAL7:IPC1 10 strains were grown under Ipc1-repressing (glucose) or Ipc1-inducing (galactose) conditions, and differentially expressed bands were identified in a polyacrylamide gel. Four bands that were up- and downregulated when Ipc1 was up- and downregulated, respectively, as compared with the corresponding WT grown on glucose or galactose, were selected. Three bands showed no homology when their sequences were blasted in the GenBank database (data not shown). Interestingly, one fragment (Figure 2a, black arrow) produced 100% homology with inhibitory protein 1 (IPI) (GenBank accession numbers AF180107, AF180108, and AY101600). This protein and the corresponding gene were previously isolated from C. neoformans by our group using different methods (unpublished observations). IPI is a cytoplasmic factor that is secreted into the growth medium, and it inhibits phagocytosis of C. neoformans by AMs (29). We renamed the previously isolated IPI1 antiphagocytic protein 1 (App1). The finding that Ipc1 modulation regulates the gene expression of APP1 was then validated by RT-PCR using specific primers against the APP1 gene and by Western analysis using anti-App1 polyclonal antibodies. Indeed, App1 was significantly up- and downregulated when Ipc1 expression was modulated as compared with the WT cells (Figure 2b).
**APP1** is a unique gene of *C. neoformans*. The sequence analysis obtained from the H99 genomic and cDNA (GenBank accession number AF180108) revealed the presence of two intronic sequences of 78 and 63 bp, producing an ORF of 543 nucleotides encoding for 181 amino acids. A putative TATA box (TATAAAA) was identified at position −72 and a possible CAT box (CAAAT) at position −10 from the ATG start site. A potential polyadenylation site (ATAAAA) was identified at position +177 from the stop codon. Sequence analysis of **APP1** revealed no significant homology with other DNA or proteins in the GenBank databases. Analysis of App1 amino acid sequences revealed the presence of five recognition sequence motifs, identified by Prosite Patterns (30). These predicted motifs comprised five putative phosphorylation sites (four for protein kinase C and one for tyrosine kinase), three myristoylation sites, and one glycosylation site. It appears, however, that a posttranslational modification is not required for App1 activity, since recombinant App1 protein produced using a prokaryotic expression system is active (see below). Whether a posttranslational modification(s) may modulate App1 activity awaits further studies. No other sequence information was obtained from homology searches.

**Figure 2**

(a) DD-RT-PCR in WT and GAL7:IPC1 10 strains using dT-A, -C, and -G with AP1 and AP2 primers. The number 1 denotes WT-glucose; 2, GAL7:IPC1-glucose; 3, WT-galactose; and 4, GAL7:IPC1-galactose. The black arrow indicates the App1 band, which is downregulated when Ipc1 is downregulated (no. 2) and upregulated when Ipc1 is upregulated (no. 4), with no significant changes in WT-glucose (no. 1) or WT-galactose (no. 3). (b) Ipc1 activity, RT-PCR, and Western blot of App1 in *C. neoformans* WT H99 and GAL7:IPC1 strains grown on glucose and galactose medium. **APP1** expression is significantly regulated by Ipc1 modulation. Actin gene was used as a control. Two independent GAL7:IPC1 strains were used for RT-PCR (nos. 10 and 13).
Disruption of the APP1 gene and its role in phagocytosis. To determine the role of the APP1 gene in the regulation of phagocytosis of C. neoformans, we disrupted the APP1 gene using the p∆app1/ΔADE2 plasmid cassette. Out of forty ADE2 transformants, two transformants showed that the APP1 ORF had been replaced by the ΔADE2 gene. Transformant 31 showed a double crossover event (Figure 3b). The homologous recombination was then confirmed by PCR using two primers flanking the upstream and downstream sequence of APP1 ORF (data not shown). Transformant 31 was chosen and named the Δapp1 mutant strain (Figure 3). Subsequently, the absence of APP1 mRNA and App1 protein in the Δapp1 mutant strain was confirmed by Northern (data not shown) and Western (Figure 3c) blotting. Reconstitution of the Δapp1 mutant was performed by the reintroduction of the APP1 gene into the Δapp1 31 strain, and transformants were analyzed by Southern and Western blotting. As shown in Figure 3b, the APP1 gene was reintroduced in the Δapp1 locus, and production of App1 protein was restored (Figure 3c). The characterization of the Δapp1 strain revealed that the absence of App1 did not affect capsule formation, melanin production, and growth at 30°C or 37°C at either pH 7.0 or 4.0 (data not shown).

Interestingly, we found that App1 protein is secreted extracellularly, in the growth medium of C. neoformans, demonstrating that App1 is a secreted protein (Figure 3d). App1 secretion was absent in the Δapp1 mutant and was restored in the Δapp1Rec strain (data not shown). Since App1 was found in the growth medium of C. neoformans cultures, we wondered whether App1 would be secreted in vivo during the infection by C. neoformans. Therefore, we tested whether phagocytosis of C. neoformans was affected by the absence of App1. C. neoformans WT H99, Δapp1, and Δapp1Rec strains were incubated with AMs, and the phagocytic indexes were determined. As shown in Figure 4a, the lack of App1 increased attachment (1.8-fold) and ingestion (3.4-fold) of yeast cells by AMs as compared with the WT strain (P = 0.01). Reintroduction of the APP1 gene (Δapp1Rec) restored the WT phenotype (Figure 4a). Therefore, App1 is a novel cryptococcal protein that exerts a unique and specific function in the regulation of phagocytosis of C. neoformans.

Production of App1 recombinant proteins and effect of App1 treatment on phagocytosis of C. neoformans. Since the above studies suggested a role for App1 in phagocytosis, it became important to determine the effect(s) of the protein on phagocytosis directly. App1 recombinant protein was therefore produced by expressing C. neoformans App1 cDNA from serotype A strain H99 into a prokaryotic expression system, as described in Methods. Clarified cell lysate obtained from the expression of pBAD-His-App1 was first loaded to a HiTrap chelating HP column loaded with nickel and then to a HiTrap Q chromatography column. Fractions from each column were analyzed by Western blotting and silver staining (data not shown), and the fraction with the highest ratio of
App1 to contaminating proteins (Q54) was used for cellular treatments. The lysate from control cells expressing pBAD-His (mock) vector was subjected to an identical purification protocol, and the same fraction collected from the App1 purification was also collected from the control lysate. Silver staining analysis confirmed that the pattern of residual contaminating proteins in the purified App1 preparation used for cellular treatments was identical to the protein pattern in the respective control sample (data not shown). The concentration of App1 was determined by the difference in the protein concentration between the fraction containing App1 and the one obtained from control cells. As can be seen from Figure 4a, addition of App1 significantly decreased phagocytosis of the Δapp1 strain in a dose-dependent manner, whereas no difference in phagocytosis was observed when the control fraction was used at the same concentration (Figure 4b). This antiphagocytic effect was also observed when C. neoformans WT strain H99 was tested. Indeed, as for the Δapp1 strain, addition of App1 decreased both attachment and ingestion of the WT strain in a dose-dependent manner, whereas treatment with the control preparation showed no effect on phagocytosis (Figure 4c). Therefore, by using both genetic and pharmacological approaches, these results indicate that App1 exerts a novel function in the regulation of phagocytosis of C. neoformans by AMs.

We also tested whether the addition of App1 would inhibit phagocytosis of other yeast cells, such as C. albicans. We found that App1 inhibited phagocytosis of C. albicans by affecting both attachment and ingestion (data not shown). Finally, we tested whether addition of App1 would have a toxic effect on AMs. After pharmacological treatment, cellular toxicity was evaluated by adding trypan blue to the AMs. We found no significant difference on the viability of AMs between App1- versus control-treated cells (data not shown).

Analysis of phagocytosis of C. neoformans WT H99, GAL7:IPC1, Δapp1, IPC1Rec, and Δapp1Rec strains by AMs was also performed ex vivo. Mice were infected intranasally with the above strains, and two hours after inoculation AMs were collected by BAL, and phagocytic indexes were determined by microscopic observation. As expected, we found that the ex vivo phagocytosis is more efficient than the in vitro phagocytosis (more yeast cells are found attached or ingested by AMs in a 2 hour period). Importantly, however, the difference in phagocytic indexes among the strains obtained ex vivo was similar.

### Figure 4
App1 regulates phagocytosis of C. neoformans. (a and b) Absence of App1 (Δapp1) increases attached and ingested cells by AMs. Reconstitution of APP1 gene (Δapp1Rec) as well as treatment with App1 recombinant protein restores the WT phenotype (a) as compared with the control (b). (c) App1 treatment decreases phagocytosis of C. neoformans WT cells. (d) Ex vivo phagocytosis. Downregulation of Ipc1 and absence of App1 increase phagocytosis of fungal cells by AMs after 2 hours of infection as compared with the WT strain. IPC1Rec and Δapp1Rec strains reconstitute the WT phenotype. (e) App1 does not inhibit phagocytosis of antibody-coated erythrocytes. (f) Treatment with App1 inhibits phagocytosis of iC3b/IgM-IgG–coated erythrocytes. WT, WT C. neoformans H99 strain; Δapp1, C. neoformans lacking App1 protein; Δapp1Rec, Δapp1 reconstituted strain; IPC1Rec, Ipc1 reconstituted strain; App1 1x, 8 ng of App1 recombinant protein plus 14 ng of contaminating proteins; App1 2x, 3x, 4x, and 5x, two-, three-, four-, and fivefold App1 1x concentration; control 1x, 14 ng of contaminating proteins; control 2x, 3x, 4x, and 5x, two-, three-, four-, and fivefold control 1x concentration.
to that obtained in vitro. Indeed, the phagocytic indexes for attached and ingested yeast cells for GAL7:IPC1 were increased by 1.9- and 2.4-fold, respectively, and the phagocytic index for attached and ingested yeast cells for Δapp1 were increased by 2.1- and 3.1-fold, respectively, as compared with the WT H99 strain (Figure 4d). This difference in phagocytosis indexes is similar to that described in Figure 1 for the GAL7:IPC1-glucose strain and in Figure 4a for the Δapp1 strain. We found no significant differences among the ex vivo phagocytic indexes of WT H99, IPC1Rec, and Δapp1Rec strains (Figure 4d).

To investigate the molecular mechanism(s) by which App1 inhibits phagocytosis, we tested whether App1 would inhibit phagocytosis of antibody-coated erythrocytes and complement/antibody-coated erythrocytes. We found that whereas App1 did not inhibit attachment or ingestion of IgM/IgG-coated erythrocytes (Figure 4e), it did significantly inhibit both attachment and ingestion of iC3b-IgM/IgG–coated erythrocytes (Figure 4f) in a dose-dependent manner (one to four times). Most interestingly, phagocytosis of iC3b-IgM/IgG–coated erythrocytes was not further inhibited by addition of the highest concentration of App1 (five times) (Figure 4f). These results clearly suggest that App1 blocks the complement- and not the antibody-mediated phagocytosis of the iC3b-IgM/IgG–coated erythrocytes.

Phagocytosis assay and App1 pharmacological treatment were performed at least twice and using AMs isolated from two mouse models (see Methods). No significant difference was found in the phagocytic index between AMs collected from either mouse model. The data presented are the geometric means ± SDs of phagocytic indexes using AMs from Tg26 mice.

Ipc1-App1 pathway regulates virulence in two different immunodeficient murine animal models. Since both Ipc1 and App1 inhibit phagocytosis of C. neoformans by AMs, we investigated whether they regulate pathogenicity of C. neoformans in a murine animal model immunodeficient for complement C5 (A/Jcr), when the lung is used as the port of entry. Mice were infected intranasally with C. neoformans WT H99, GAL7:IPC1, Δapp1, IPC1Rec, and Δapp1Rec strains, and survival of the mice was monitored. The App1 protein is absent in the Δapp1 strain (Figure 3), whereas Ipc1 activity is indeed downregulated in the animal (data not shown), since glucose, instead of galactose, is available as a carbon source. The average survivals of GAL7:IPC1- and Δapp1-infected mice were 25.4 ± 5.2 and 21.7 ± 2.11 days, as compared with 14.9 ± 3.31 days for WT-infected mice (P = 0.002 and 0.01, respectively) (Figure 5a). Interestingly, survival of Δapp1-infected mice significantly decreased at a later phase of infection (26th day), whereas mice infected with the GAL7:IPC1 strain showed increased survival (40% GAL7:IPC1 versus 5% Δapp1, respectively). The IPC1Rec and Δapp1Rec strains showed average survivals of 18.8 ± 3.2 days and 17.1 ± 2.5 days, respectively, which are not significantly different from the average survival observed with WT-infected mice (P > 0.05). These results indicate that downregulation of Ipc1 or absence of App1 produces a strain less virulent than the WT in this mouse model.

Since infection by C. neoformans mainly occurs in immunocompromised hosts, such as patients with AIDS in whom T and NK cell number and function are significantly impaired (31–33), we investigated whether Ipc1 and App1 would have a role in the pathogenicity of C. neoformans in a murine animal model immunodeficient for T and NK cells. For this experiment, we used Tg26 mice, which lack functional T and NK cells, mimicking the condition found in patients with AIDS (19). We infected Tg26 mice intranasally and followed their survival. The average survival of GAL7:IPC1-infected Tg26 mice was 20.14 ± 2.95 days, which was significantly longer than the 16.21 ± 1.76 days for H99-infected mice (P = 0.01) (Figure 5b). Surprisingly, Tg26 mice infected with the Δapp1 mutant strain showed decreased survival (13.5 ± 0.51) as compared with the WT H99 strain (P = 0.03) (Figure 5b). The average survivals of IPC1Rec- and Δapp1Rec-infected mice
were 17 ± 2.2 and 15.85 ± 1.79 days, similar to the average survival of 16.21 ± 1.76 days observed with the WT-infected mice (P > 0.05). These results indicate that the absence of App1 produces a strain more pathogenic than the WT in mice with severe NK and T cell immunodeficiencies.

To determine whether the dissemination of yeast cells from the lung to the brain was affected by downregulation of Ipc1, by the absence of App1, and/or by the immune response of the host, lungs and brains were removed and assayed for viable C. neoformans. The number of GAL7::IPC1 and Δapp1 cells recovered from the lungs of A/Jcr-immunodeficient mice was significantly lower than the number of recovered WT cells (P = 0.02). Additionally, both GAL7::IPC1 and Δapp1 strains showed a decreased dissemination to the brain as compared with the WT strain, which was statistically significant at days 15 and 20 of infection (P = 0.01 and P = 0.015, respectively) (Figure 6, top panels). Therefore, Ipc1 and App1 are important for C. neoformans to establish infection in the brain of an A/Jcr-immunodeficient mouse model.

In Tgε26-immunocompromised mice, however, the absence of App1 produced a strain that proliferated at a higher rate in the lung and disseminated readily to the brain as compared with the WT strain (P = 0.002) (Figure 6, bottom panels). Conversely, we found that Tgε26 mice infected with the GAL7::IPC1 strain showed a drastic decrease of fungal dissemination to the brain as compared with those infected with the WT strain (P = 0.001) (Figure 6, bottom panels). As expected, these data obtained with tissue-burden culture studies corroborate those obtained with survival studies, suggesting that the absence of App1 produces a strain more pathogenic than the WT in mice with severe immunodeficiency, and that Ipc1 may regulate pathogenicity of C. neoformans through mechanisms other than modulation of App1. Both survival and tissue-burden culture studies were performed by using a second and independent GAL7::IPC1 13 and Δapp1 6 transformant, and no significant difference was found versus the corresponding previously used mutant strain (data not shown). Additionally, both IPC1Rec- and Δapp1Rec-infected mice showed survival and fungal cell dissemination similar to those observed with the WT-infected mice (Figures 5 and 6), showing that the WT phenotype(s) have been restored by reintroducing the gene(s) back into the corresponding knockout mutants.

Histopathology. To analyze the effect of downregulation of Ipc1 and/or loss of App1 on the host inflammatory response in vivo, we studied histology of lungs and brains during the infection. The immunodeficient mouse models were infected with C. neoformans WT H99, GAL7::IPC1, Δapp1, IPC1Rec, and Δapp1Rec strains, and at days 5 and 15 after inoculation for the A/Jcr mouse model or days 6 and 13 after inoculation for the Tgε26 mouse model, lungs and brains were collected, fixed in 10% formalin, and embedded in paraffin, and sections were stained with hematoxylin and eosin to visualize the host inflammatory response, PAS to identify the fungal cell wall and body, and mucicarmine (data not shown) as additional staining specific for the C. neoformans capsule (34). Selected sections were also subjected to immunohistochemical analysis using anti-CD68 antibody to identify macrophages.

In the mouse model with CS deficiency (A/Jcr), an intense PAS-positive deposition due to mucus hyperplasia/metaplasia (stained red by PAS) at day 5 of the infection in bronchi and terminal bronchioles was found in lungs infected with GAL7::IPC1 (Figure 7c) or Δapp1 (Figure 7d) strains. Interestingly, this phenomenon was not present in bronchi and bronchioles not containing yeast cells or in lungs infected with the WT strain (Figure 7b), normal uninfected lung (Figure 7a), or IPC1Rec- and Δapp1Rec-infected mice (data not shown). The mucus hyperplasia/metaplasia was still detectable at day 15 of infection, although it was much less extensive (data not shown). At day 15 of the infection, the host cellular response was dramatically activated. In particular, the
inflammatory response against the WT strain was comprised of polymorphonuclear cell infiltrates, such as neutrophils (orange arrowheads), with few eosinophils (yellow arrowheads), lymphocytes, and macrophages (green arrowhead, Figure 7f). Interestingly, the host cellular infiltrates in lung infected with the GAL7:IPC1 strain were also represented by neutrophils but with a large majority of macrophages and few lymphocytes and eosinophils (Figure 7g). Infiltrates in lungs infected with the ∆app1 strain also contained neutrophils but with a large number of lymphocyte infiltrations (violet arrowheads), with few macrophages and eosinophils (Figure 7h). Lung infected with either IPC1Rec or ∆app1Rec strains showed a cellular infiltration similar to that observed with the WT strain (data not shown).

In the mouse model with impaired T and NK cells (Tgε26), no mucus hyperplasia/metaplasia was found at day 6 of the infection in lungs infected with either WT (Figure 7j), GAL7:IPC1 (Figure 7k), ∆app1 (Figure 7l, IPC1Rec, or ∆app1Rec strains (data not shown). Interestingly, a local dissemination of WT fungal cells inside macrophages was found in lymphatic vessel and fat tissue in proximity of the lung (green arrowheads in inset of Figure 7j). This phenomenon was more evident with the ∆app1 mutant, and macrophages, loaded with this mutant strain, infiltrated not only fat tissue but also pulmonary lymph nodes (green arrowheads in inset of Figure 7l). This local dissemination was not observed in lung sections infected with the GAL7:IPC1 strain (Figure 7k). In contrast to the A/Jcr model, day 13 of infection the inflammatory response in the Tgε26 mouse model against the WT strain was comprised mostly of macrophages with few lymphocytes, whereas neutrophils were absent in the cellular infiltrates (Figure 7n). The recruitment of macrophages was more evident in lungs infected with the GAL7:IPC1 strain, where an intense interstitial infiltration of macrophages represented the major host inflammatory response against this strain (Figure 7o). Interestingly, in lung infected with ∆app1 strain, a dramatic dissemination of fungal cells in fat tissue and local lymph nodes was found. As shown in Figure 7p, fat tissue and residual lymphoid tissue between two bronchi (upper left and lower left) were completely replaced by C. neoformans ∆app1 cells.

Histology of brain tissues from both animal models infected with WT, GAL7:IPC1, and ∆app1 strains corroborated the results obtained with tissue-burden studies. Animals infected with GAL7:IPC1 strain showed a decreased fungal dissemination as compared with the WT strain in both animal models (Figure 7, s and w versus r and v). Animals infected with ∆app1 mutant showed a decreased dissemination in the A/Jcr model (Figure 7c), whereas in Tgε26, the mutant strain reached the brain as early as 6 days after infection (inset in Figure 7x) and more dramatically at 13 days (Figure 7x).

These results clearly show that the host lung inflammatory response against C. neoformans between the A/Jcr and Tgε26 mouse models is different, with a prevalent neutrophil infiltration in the A/Jcr model and a prevalent macrophage infiltration in the Tgε26 model. These studies indicate that whereas dissemination of the GAL7:IPC1 strain is impaired, the ∆app1 strain readily disseminates within macrophages to fat and lymphoid tissues found in the mediastinum area. Histology of lung and brain was performed using two animals for each strain (WT, GAL7:IPC1, ∆app1, IPC1Rec, and ∆app1Rec strains). No significant differences were found between the animals infected with the same strain. The cellular infiltrates observed with IPC1Rec and ∆app1Rec strains were similar to those observed with the WT strain (data not shown). Macrophage involvement was demonstrated using anti-CD68 antibody and performed in all tissue sections examined (Figure 7x and data not shown).

Discussion
We previously demonstrated that regulation of Ipc1 plays a major role in pathogenicity of C. neoformans in the rabbit animal model (9). This model allows the study of the impact of a gene of interest on pathogenicity of C. neoformans during the late stage of cryptococcosis (meningoencephalitis). In the present study, we investigated the role of Ipc1 during early stages of the infection using murine animal models of cryptococcosis. Since the lung is the port of entry for C. neoformans, AMs are one of the first host defenses against the inhaled microorganism. Indeed, we found that Ipc1 regulates phagocytosis of C. neoformans by AMs, and this regulation occurs through the modulation of a newly identified protein, App1.

During the 1980s and 1990s, we had focused on the identification and purification of cytotoxic factor(s) of C. neoformans involved in the regulation of phagocytosis of yeast cells by AMs. These early studies resulted in the partial isolation and purification, from crude cytoplasmic extract, of an approximately 20-kDa protein as a cryptococcal factor that specifically inhibits phagocytosis (29). Rabbit polyclonal antibodies were raised against this cytoplasmic factor and used to screen a C. neoformans cDNA library. A putative gene encoding for an approximately 20-kDa protein was identified and sequenced from both serotypes A and D of C. neoformans (unpublished observations; GenBank accession numbers AF180107, AF180108, and AJ101600). Using polyclonal antibodies, it was shown that this cytoplasmic factor is secreted extracellularly in culture medium of C. neoformans (29) (Figure 3). These results were corroborated by the fact that this protein was found in serum of patients with AIDS who had disseminated cryptococcosis (Figure 3d) (35). In this study, we isolated by DD-RT-PCR the same gene isolated previously, whose expression is regulated by the level of Ipc1 activity. This gene was named APP1 for antiphagocytic protein 1, and through a genetic, biochemical, and immunological approach we characterized the function of App1 on phagocytosis and virulence of C. neoformans. The APP1 gene appears to be regulated at the transcriptional level by modulation of Ipc1 activity (Figure 2b). In addition, DD-RT-PCR identified three other fragments to be regulated by IPC1 expression (data not
Figure 7
Histopathology of lungs and brains from animal models. (a–d, i–l, q–t, and u–x) PAS-positive staining. (e–h and m–p) Hematoxylin and eosin staining. Histopathology of lung from A/Jcr (a–d and e–h) and Tgε26 (i–l and m–p) is shown. Normal lung (a, e, i, and m) and lungs infected with C. neoformans WT (b, f, j, and n), GAL7:IPC1 (c, g, k, and o), and Δapp1 (d, h, l, and p) at 5 (a–d) and 15 (e–h) days and at 6 (i–l) and 13 (m–p) days after infection are shown. In the A/Jcr mouse model, a mucus hyperplasia/metaplasia (PAS-positive material) is present in lungs infected with the GAL7:IPC1 (c) and Δapp1 (d) strains (red arrowheads, c and d), but not in the lung infected with the WT strain (b). At day 15 of infection (e–h), intense neutrophil recruitment is observed in lung infected with the WT strain (orange arrowheads and orange circle, f), whereas neutrophils and macrophages are found in lung infected with the GAL7:IPC1 strain (orange arrowheads and green arrowheads, g) or neutrophils and lymphocytes are found in lung infected with the Δapp1 strain (orange arrowhead and violet arrowheads, h). At day 6 of infection in the Tgε26 mouse model (i–l), no mucus hyperproduction was observed (j–l and data not shown). Instead, dissemination of C. neoformans within macrophages in brown fat and lymphoid tissue was observed with the WT strain (green arrowhead, j) and, more pronounced, with the Δapp1 strain (green arrowhead and green circle, l) but not with the GAL7:IPC1 strain (k). At day 13 of infection (m–p), the inflammatory response against WT, GAL7:IPC1, and Δapp1 is mainly represented by macrophages (green arrowheads, n–p). The Δapp1 yeast cells are found mainly in brown fat (p). Histopathology of brains from A/Jcr (q–t) and Tgε26 (u–x) mice at days 15 and 13 of infection, respectively. Normal brains (q and u) and brains infected with WT (r and v), GAL7:IPC1 (s and w), and Δapp1 (t and x) are shown. C. neoformans cells were found in brains infected with the WT strain (r and v), whereas no yeast cells were found in brain sections examined from mice infected with the GAL7:IPC1 strain (s and w). The Δapp1 yeast cells were not found in brain sections examined from A/Jcr brain (t), whereas numerous Δapp1 cells were found in Tgε26 infected brain (x). Additionally, an Δapp1 yeast cell was found inside a macrophage (stained brown with anti-CD68 antibody) in a blood vessel of brain tissue at day 6 of infection (inset in x). (j, l, p, and x) In the insets, black arrowheads indicate yeast cells inside macrophages. (f and g) Yellow arrowheads indicate eosinophils. (c, d, f–h, and n–p) Insets are magnifications of small squared area. (r) Inset represents PAS staining of a different brain section. (x) Inset represents immunohistochemistry with anti-CD68 antibody for macrophage staining. Histology studies were also performed using IPC1Rec and Δapp1Rec strains, showing a phenotype similar to that observed with the WT strain (data not shown). Magnification, ×40. The images are representative of three different organ sections.
shown), but no homology to any other gene sequences was found. We focused our attention on App1 (Figure 2a) because it was already isolated and shown to exert a specific function on the phagocytosis of *C. neoformans*. Since Ipc1 transcriptionally modulates the expression of APP1, it is likely that this regulation occurs at the promoter region of the APP1 gene, although Ipc1 may also regulate the stability of APP1 mRNA. A search of putative transcription factors present in the APP1 promoter (Web promoter scan service provided by the Bioinformatics and Molecular Analysis Section at NIH, http://molbio.info.nih.gov/molbio/db.html) showed significant hits for the following transcription factors: AP-2, CREB, and ATF. In our studies, we found that modulation of Ipc1 regulates the level of diacylglycerol (DAG) (unpublished observation) (36). Interestingly, it has been shown that DAG may exert a regulatory effect on the transcription factor ATF/cAMP-responsive element in RINm5F cells (37). Since APP1 promoter contains this putative signal, it is reasonable to hypothesize that transcriptional activation of APP1 is mediated through the lipid regulated by Ipc1.

Because the native App1 protein previously isolated was involved in the inhibition of phagocytosis, we disrupted the APP1 gene in *C. neoformans* and studied whether phagocytosis would be affected by the absence of App1. We found that, indeed, phagocytosis of *C. neoformans* increased when App1 protein was absent (Figure 4a). Since App1 does not affect other cryptococcal factors known to influence phagocytosis, such as the capsule, it is proposed that App1 regulates phagocytosis through a specific and novel molecular mechanism. Addition of recombinant App1 protein or reconstitution of APP1 gene in the ∆app1 strain restores the ∆app1 phenotype nearly at the WT level (Figure 4a). App1 treatment further reduces phagocytosis of the WT strain (Figure 4c) by decreasing both attachment and ingestion of yeast cells by AMs, although the inhibitory effect of App1 seems more pronounced on the ingestion process (Figure 4c). The observation that App1 also inhibits phagocytosis of *C. albicans* (data not shown) suggests that App1 may act by blocking a common receptor responsible for attachment and internalization of these yeasts. The fact that App1 does not block phagocytosis of antibody-coated erythrocytes, whereas it does inhibit the phagocytosis of complement/antibody-coated erythrocytes, clearly suggests that App1 exerts its effect through the inhibition of complement-mediated phagocytosis, which is a common molecular mechanism by which fungal cells are phagocytosed. Indeed, several studies have suggested that phagocytosis of *C. neoformans* is primarily mediated through complement receptors (CR1, CR3, and CR4), although other mechanisms are also involved, such as antibody-mediated (anti-GXM) and mannose-mediated mechanisms (38–41). Among the complement receptors, CR3 (CD11b/CD18) plays a major role in the attachment and internalization of *C. albicans* (42), Blastomyces dermatitidis (43), *C. neoformans* (40, 44, 45), and bacteria (46) by the host’s phagocytic cells. Since our results show that App1 blocks iC3b-mediated phagocy-
tosis, it is reasonable to hypothesize that App1 inhibits the internalization of *C. neoformans* by blocking the interaction of fungal cells with the complement receptor. It remains to be established which complement receptor(s) are most affected by App1 action.

The provirulence effects of Ipc1 are mediated through the regulation of various factors, such as melanin formation, intracellular growth (9), and phagocytosis through App1 (this study). Differential modulation of these factors by Ipc1 could be exerted at different stages of the infection (i.e., initiation, dormancy, reactivation, dissemination, proliferation). Due to its antiphagocytic role, App1 may be produced by *C. neoformans* during early stages of the infection (initiation-dissemination). Once inhaled, *C. neoformans* cells are phagocytosed by AMs, and this internalization is required for effective killing of the microorganism, when the host cellular response is functional. In a mouse model deficient for complement C5, in which the immune response is still cell mediated, we found that downregulation of Ipc1 and absence of App1 decrease virulence. However, *C. neoformans* is a fungal pathogen that primarily afflicts immunocompromised subjects such as those affected by HIV, where the cell-mediated immune response (T and NK) is significantly impaired. In these patients, phagocytosis might not represent an effective mechanism of defense by the host, when the activation of T and/or NK cells and, thus, the production of activated macrophages are significantly reduced (6, 47–49). Moreover, in the case of facultative intracellular pathogens, such as *C. neoformans*, for which intracellular growth can be a favorable condition, phagocytosis may represent a successful strategy for better survival in the host. Indeed, when we investigated the effect of absence of App1 in an immunocompromised mouse model deficient for T and NK cells, we found that virulence (Figure 5b) and dissemination of *C. neoformans* infection were significantly enhanced (Figure 6, bottom panels). As a result, absence of App1 protein significantly increases the number of yeast cells recovered from the brain. These results indicate that lack of App1 favors the internalization of *C. neoformans* into nonactivated macrophages and the dissemination of fungal cells to the brain when T and NK cells are not functional.

The host inflammatory response to the *C. neoformans* infection in A/Jcr and Tgε26 mice is different. First, in the A/Jcr model, a mucus hyperplasia/metaplasia is present in bronchi and bronchioles of the lung infected by GAL7/IPC1 or ∆app1 strains (Figure 7, c and d), and it seems to be stimulated by the nearby presence of the GAL7/IPC1 or ∆app1 cells, since it is absent in Airways that do not contain yeast cells. It has been reported that mucus contains mucin (MUC7) and histatin-5 (Hsn-5), two peptides that, among their spectrum of antimicrobial activity, display a potent killing effect against a variety of fungi, including *C. albicans* and *C. neoformans* (50–53). This hyperproduction of mucus may represent a host defense mechanism against inhaled microorganisms, and, interestingly, it appears to be stimulated particularly by less virulent strains, since it is less evident in lung infected with
the WT strain (Figure 7b). In recent years, studies have shown that activation of the Th2 response leads to mucus hypersecretion (54). The A/J mice, and presumably also the A/Jcr mice, are prone to develop a Th2 response due to the downregulation of IL-12 in the absence of C5 (26–28). This could explain why the mucus hyperplasia/metaplasia is observed in the A/Jcr model and not in the Tgε26 mice. However, it does not completely explain why the mucus hypersecretion is not stimulated in the A/Jcr mice infected with the WT strain, suggesting that this phenomenon is a complex process, also determined by the characteristics of the pathogen(s). Second, the cellular inflammatory response in the A/Jcr mouse model is mainly represented by neutrophils, whereas in the Tgε26 mouse model it is mainly represented by macrophages (Figure 7; compare f, g, and h with n, o, and p, respectively). This difference in the inflammatory response may account for the difference we observed in virulence among the WT, GAL7:IPC1, and Δapp1 strains, especially in the Tgε26 mice. Although macrophages are taking up more GAL7:IPC1 cells than the WT, this mutant strain shows a profound defect in intracellular growth (due to its defect on growth at pH 4.0) and on melanin production (9). In this case, a macrophage response may be able to partially control the GAL7:IPC1 dissemination. In the case of infection by Δapp1, however, the macrophage response in Tgε26 mice is clearly unable to control the dissemination. The Δapp1 strain is more internalized by macrophages than the GAL7:IPC1 mutant; it does not have a defect on growth at low pH or an alteration of melanin production. Its dissemination seems to be determined by the host’s immune response. In a mouse model deficient for C5 (A/Jcr), macrophages and lymphocytes are recruited into the lung decreasing fungal dissemination to the brain (Figures 6 and 7). In contrast, in a mouse model deficient for T and NK (Tgε26), the dissemination of the Δapp1 strain occurs very early during the infection (day 6) into extrapulmonary tissue (Figure 7, l and p) and into the brain (inset in Figure 7x). Lack of App1 seems also to favor recruitment of lymphocytes in the lung of A/Jcr mice (Figure 7h) as compared with the WT (Figure 7f), suggesting that App1 may exert an inhibitory effect on other cells involved in the host cellular immunity, such as lymphocytes.

The physiological mechanism by which this dissemination occurs remains to be elucidated. However, these studies clearly suggest that, without App1, C. neoformans is more easily taken up by macrophages, which, in mice with an intact T and NK cellular response, can control/kill the yeast. In T-NK–deficient mice, the macrophage may allow the yeast to be “protected” as it migrates from the lung to the brain. In other words, the macrophage can act as a “Trojan horse” for the yeast to reach other tissues (Figure 7f) and the central nervous system (Figure 7x), and this mechanism of dissemination is also observed with the WT strain (Figure 7f) and in previously reported observations (55). On the other hand, impairment of a cellular response could favor the yeast growth inside the macrophages, inducing their lysis. The released yeast cells in the extracellular matrix can then reach the central nervous system through the blood stream. Further studies are needed to investigate these potential mechanisms.

In the same immunocompromised conditions (Tgε26), downregulation of Ipc1 resulted in a dramatic reduction of the dissemination of C. neoformans cells to the brain (Figure 6, bottom) or other tissues (Figure 7k), suggesting that the effect of the modulation of Ipc1 on virulence of C. neoformans is not only mediated through the regulation of App1 and phagocytosis. This hypothesis is further supported by the results obtained in the A/Jcr model in which the antipathogenic effect obtained by downregulating Ipc1 was more pronounced than that observed when App1 protein was absent. Indeed, we previously demonstrated that Ipc1 regulates other downstream effects, such as melanin production and intracellular growth, and these factors are important for the outcome of the infection (9, 36, 56, 57).

In conclusion, this study defines a novel function for Ipc1 in the regulation of pathogenicity of C. neoformans during early stages of the infection. In particular, we identify that modulation of Ipc1 regulates the level of a novel protein, App1. Biochemical characterization of App1 reveals its function as an antiphagocytic factor. The Ipc1-App1 pathway regulates phagocytosis of C. neoformans by AMs and virulence in the murine animal models of disseminated cryptococcosis by potentially affecting the numbers of the intra- versus extracellular yeast cells. Importantly, we show that this trait assumes a crucial role for the development of cryptococcosis in immunocompromised hosts.

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