Role of Glucose in the Expression of *Cryptococcus neoformans* Antiphagocytic Protein 1, App1

Virginia Williams¹ and Maurizio Del Poeta¹,²,³*

Departments of Biochemistry & Molecular Biology¹ and Microbiology & Immunology² and Division of Infectious Diseases,³ Medical University of South Carolina, Charleston, South Carolina 29425

Received 5 October 2010/Accepted 4 January 2011

The cryptococcus-specific protein antiphagocytic protein 1 (App1) regulates *Cryptococcus neoformans* virulence by controlling macrophage-driven fungal phagocytosis. This is accomplished through complement receptors (CR), specifically CR3. When inhaled, *C. neoformans* can cause a life-threatening meningoencephalitis in immunocompromised patients. Because glucose starvation can significantly change the gene expression and virulence of *C. neoformans* and because App1 is critical for phagocytosis in the lung—a low-glucose environment—we investigated the role of glucose in App1 expression. We found that App1 was upregulated dramatically under low-glucose conditions, and it was upregulated when *C. neoformans* cells were incubated in bronchoalveolar lavage (BAL) fluid, serum, and cerebrospinal fluid, which are low-glucose environments. Characterization of App1’s regulation based on mammalian lung physiology revealed that App1 is upregulated via both increases in transcription and increases in mRNA stability. Our data provide new insights regarding *C. neoformans* adaptations to low-glucose environments.

*Cryptococcus neoformans* is an environmental yeast that causes life-threatening meningoencephalitis, primarily in immunocompromised patients (10). *C. neoformans* infection begins with inhalation of spores or desiccated yeast cells into the host lung. *C. neoformans* can then be cleared from the lung or contained in an immunocompetent subject, but in an immunocompromised patient, the yeast cells will replicate and eventually disseminate to the central nervous system through the bloodstream (10). Interestingly, the facultative intracellular pathogen *C. neoformans* can grow and replicate inside host macrophages as well as in extracellular spaces (13, 14, 25).

Antiphagocytic protein 1 (App1) is a virulence factor of *C. neoformans* that inhibits macrophage-mediated fungal phagocytosis in a dose-dependent and complement-mediated manner (26). Specifically, App1 inhibits phagocytosis via complement receptor 3 (CR3) (42), under the control of inositol phospholipid ceramide synthase 1 (Ipc1) (28), an enzyme of the sphingolipid pathway that produces complex sphingolipids and diacylglycerol (DAG) (27). Mare et al. showed that Ipc1 regulates App1 gene expression through DAG (28). Additional studies showed that DAG activates App1 gene transcription via transcription factor 2 (Atf2) activation: deletion of the ATF2 gene or mutation of the ATF cis-acting element in the APP1 promoter completely halts App1 expression controlled by Ipc1 or DAG (28, 43). Although these studies indicate a role for App1 in regulating fungal pathogenicity, the precise environmental signal or stress that controls this protein is unknown.

Other *C. neoformans* virulence factors have been shown to be regulated by mammalian physiological conditions that are encountered in the lung (39, 49). For example, melanin production is induced during glucose starvation due to the low-glucose lung environment, and capsule production is stimulated by iron deprivation and by a physiological concentration of CO₂ (5%). Mating is induced by nitrogen starvation (1, 48), and the sphingolipid glucosylceramide is localized mainly on the cell surface at neutral/alkaline pH (38). Thus, fungi can interpret and respond to temperature, pH, gases, and nutrients and adapt to new environments through modified biological responses (3).

Signaling pathways involved in nutrient sensing, particularly glucose sensing, have been studied more thoroughly in *Saccharomyces cerevisiae* (9, 16, 21), and they stimulate a series of responses through both transcriptional and posttranscriptional regulation (11). In *S. cerevisiae*, sucrose-nonfermenting (Snf) proteins are involved in sugar utilization (8), and Snf5 in *C. neoformans* is required for the expression of glucose-repressed genes (23). Other Snf proteins, such as Snf1, control melanin production during glucose starvation (19) in *C. neoformans*, a mechanism shared by Gpa1 through the cyclic AMP (cAMP)-protein kinase A (PKA) pathway (1–3, 36, 49).

Because App1 regulates phagocytosis of *C. neoformans*, we hypothesize that its expression and activity are most influential in the lung at early stages of the infection, when initial interactions occur between *C. neoformans* and alveolar macrophages. The lung has significantly lower glucose levels than the blood (alveolar spaces have 1/50 the typical blood glucose concentration of 100 mg/dl) (22, 29, 30, 41), and macrophages have high rates of glucose utilization (29). Thus, during macrophage and neutrophil recruitment to the site of *C. neoformans* infection, glucose concentrations could decrease further.

We therefore studied whether App1 expression is regulated by the glucose concentration, and we report that App1 is upregulated significantly during glucose starvation when cells are grown in *vitro* and when they are incubated in human body fluids, such as bronchoalveolar lavage (BAL) fluid, serum, and...
cerebrospinal fluid (CSF). App1 expression also increases when C. neoformans cells are incubated in macrophage-conditioned medium. Finally, we provide evidence that App1 up-regulation in the presence of low glucose concentrations is due to both increased App1 transcription and increased mRNA stability.

MATERIALS AND METHODS

Strains, growth media, and reagents. C. neoformans var. grubii serotype A strains H99 and KN99 are wild-type strains. The ΔC1-Pka1 and ΔIC1/App1::LUC/ATP2::HA strains were created previously in our laboratory (see references 18 and 43, respectively). The C. neoformans Δα2p1 (1) and Δα2p5 (44) strains were kindly provided by J. Heitman, Duke University. The Δα1p, Δα1p, and Δα1p (19) strains were kindly provided by James Kronstad, University of British Columbia. All C. neoformans strains were grown on yeast extract-peptone-dextrose (YPD) medium containing 2% glucose, unless otherwise stated. In experiments in which lower glucose concentrations were used, C. neoformans strains were grown in YP medium with the stated glucose concentration. Human samples were obtained from the Clinical Microbiology Laboratory of the Medical University of South Carolina. All samples were deidentified and were received with randomly assigned numbers. The MHS (ATCC) mouse alveolar macrophage cell line was used in cell culture experiments, as indicated. MHS cells were grown in RPMI (Gibco) medium with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin, maintained at 37°C and 5% CO2 and passed every 2 to 3 days.

Production of App1 antibodies. Anti-App1 monoclonal antibodies (MAbs) were generated by immunizing BALB/c mice with glutathione S-transferase (GST-Fused) App1 emulsified with Freund’s complete adjuvant (Sigma Inc., St. Louis, MO). After two additional booster immunizations with App1 recombinant protein in Freund’s incomplete adjuvant (Sigma Inc., St. Louis, MO), sera were collected, pooled, and stored at −20°C.

RNA extraction and real-time PCR. RNA was isolated from C. neoformans cells by using an RNeasy mini kit from Qiagen (Valencia, CA). App1 cDNA was generated using 1 μg total RNA and oligo(dT) primers from a SuperScript first-strand synthesis reverse transcription kit (Invitrogen, Carlsbad, CA). Real-time PCR was conducted on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). The real-time PCR was carried out using a 25-μl reaction mix containing cDNA, SYBR green (Bio-Rad, Hercules, CA), 1 μl each of forward and reverse primers (10 μM), and H2O. The reaction cycle conditions were as follows: 95°C for 3 min for denaturation, 40 cycles of 95°C for 10 s for denaturation and 58°C for 45 s for annealing, 95°C for 1 min, and 58°C for 1 min, followed by a melting curve analysis. Data were analyzed using MyIQ software (Bio-Rad). All reactions were carried out in triplicate, and melt curves were analyzed to ensure that there was a single product curve for each sample.

App1 expression in human samples. Human BAL fluid, serum, and CSF samples were obtained from the Clinical Microbiology Laboratory at the Medical University of South Carolina. Samples from four different patients were pooled together and centrifuged at 3,000 rpm for 10 min, and only supernatant was used for the experiment, to ensure that no effect was due to human cells. C. neoformans wild-type cells were grown for 18 to 20 h in culture medium and then first incubated in BAL fluid for 24 h, then transferred to serum for an additional 24 h, and finally incubated in CSF for 24 h. Approximately 1010 cells were incubated in each ml of fluid, and after each incubation, cells were used for protein or RNA extraction. CFU counts were performed to check for viability. App1 expression in mouse BAL fluid. BAL fluid was obtained from CBA/J or TgEr26 mice as described by Luberto et al. (26). The protocol was approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (permit number 2019). All animal procedures were performed according to the approved protocol and every effort was made to minimize suffering. Three 4- to 6-week-old female mice were used for each mouse model. The BAL fluid was then collected 3,000 rpm for 5 min at room temperature. The supernatant was transferred to a new tube. Glucose in the BAL fluid was measured by using a GAHK-20 quantitative enzymatic reaction kit from Sigma (St. Louis, MO). Briefly, BAL fluid samples were combined with a mixture containing hexokinase, NAD, ATP, water, and...
the App1 protein level was significantly increased when cells were grown in medium with 0.06% compared to 2% glucose. To further support this observation, real-time PCR was performed to measure App1 mRNA levels in the presence of different glucose concentrations. Figure 1B shows that App1 mRNA expression in cells grown in 0.06% glucose was significantly higher than App1 mRNA expression in 2% glucose. As expected, App1 mRNA was not detected in the C. neoformans Δapp1 mutant strain.

App1 expression is dependent on glucose concentration. After observing an upregulation of App1 during glucose starvation, we hypothesized that App1 is directly affected by the glucose in the environment. To determine if the effect of glucose on App1 is dose dependent, wild-type C. neoformans H99 was grown in the presence of various glucose concentrations between 0.002% (estimated lung concentration) and 2% (standard in vitro culture concentration). As shown in Fig. 2A and B, as glucose in the medium increased, App1 protein expression decreased. Further evidence that App1 expression is directly regulated by the glucose concentration is shown in Fig. 2C, which shows that the level of App1 mRNA also decreased when the concentration of glucose in the medium increased.

We next studied the effect of incubation time on App1 expression. We thought that if C. neoformans cells were allowed to grow in the same medium for an extended period, glucose would be consumed, and as a consequence, App1 expression would increase. Thus, we monitored App1 expression at 24, 48, and 72 h of growth and found that its expression increased significantly (Fig. 2D). Interestingly, when glucose was added back after 72 h, App1 expression at 96 h decreased significantly, to a level similar to that at the original 24-h time point. As glucose was further consumed at 120 and 144 h, App1 increased again (Fig. 2D). These results suggest that glucose consumption may be a key factor in the regulation of App1 expression.

App1 expression is also regulated by galactose. We then tested whether this effect was due to an overall sugar deprivation or to consumption of a particular sugar(s). Thus, App1 expression was examined when C. neoformans cells were grown in the presence of galactose, maltose, or sucrose, in addition to glucose. We found that when C. neoformans cells were incubated in the presence of low galactose concentrations, both App1 mRNA (Fig. 3A) and App1 protein (Fig. 3B and C) expression was increased similarly, to the level observed when glucose was used as a carbon source. This effect was not observed with maltose or with sucrose (data not shown). These results suggest that App1 expression is not regulated by an overall sugar starvation but rather by a signaling pathway(s) controlled by specific sugars, such as glucose and galactose.

Effect of physiological temperature and CO2 on App1 expression. We next determined whether App1 expression is regulated by temperature and/or CO2 concentration. Thus, C. neoformans cells were grown in 2% glucose for 18 to 20 h under three different sets of conditions: (i) 30°C and 0.04% CO2, (ii) 37°C and 0.04% CO2, and (iii) 37°C and 5% CO2. Total protein and RNA were extracted and subjected to Western blotting and real-time PCR, respectively. We found that App1 expression was increased at 37°C compared to 30°C and also at 5% CO2 compared to 0.04% CO2 (Fig. 4A and B).

Interestingly, although App1 mRNA increased significantly

---

FIG. 1. App1 expression is increased under low-glucose conditions. The C. neoformans wild-type (WT) or Δapp1 mutant strain was grown in medium with 2% or 0.06% glucose for 18 to 20 h while shaking, and App1 expression was analyzed using Western blotting and real-time PCR. (A) Western blot analysis showing an increase in App1 protein at a low (0.06%) versus high (2%) glucose (Glu) concentration. (B) Real-time PCR analysis showing an increase in App1 mRNA expression in 0.06% compared to 2% glucose. *, P < 0.05 for 0.06% versus 2% glucose by the Student t test.

---

RESULTS

App1 is upregulated during glucose starvation. To determine if App1 expression is regulated by the glucose concentration, wild-type C. neoformans (H99) was grown in YP medium with 2% glucose or 0.06% glucose. As shown in Fig. 1A, glucose-6-phosphate dehydrogenase. Glucose in the sample is phosphorylated by hexokinase to form glucose-6-phosphate. The phosphorylated sugar is then converted to 6-phosphogluconate. This reaction results in the oxidation of NAD to NADH. The increase in NADH results in a change in absorbance at 340 nm and can therefore be quantified. Glucose was also added to the BAL fluid to reach a concentration of 0.002% to more closely mimic the lung conditions. C. neoformans wild-type H99 cells were counted and washed in PBS, and then 109 cells were inoculated into the BAL fluid for 18 to 20 h. During this incubation, C. neoformans cells did not grow due to a lack of glucose or very low glucose concentration. For controls, the effects of cell density on App1 expression and cell viability were examined. Cell density did not affect either App1 expression or cell viability (data not shown). After incubation, cells were washed and used for either protein or RNA extraction.

App1 expression in macrophage-conditioned medium. MHS cells were routinely passaged and maintained in RPMI medium containing 1% glucose. For this experiment, cells were washed, counted, and seeded in 25-cm2 flasks with 0, 105, 5 × 105, or 106 cells. MHS cells were incubated in 10 ml of 1% glucose medium with 10% FBS and 1% penicillin-streptomycin for 72 h at 37°C and 5% CO2. After 72 h, cells were centrifuged at 800 rpm for 5 min, and the supernatant was transferred to new tubes. One set of supernatants was used “as is,” and 1% glucose was added to a second set of supernatants before the addition of 10% C. neoformans wild-type cells. Cultures were allowed to incubate for 18 to 20 h at 30°C and 0.04% CO2 while shaking. Cultures were then centrifuged, washed, and subjected to RNA extraction and real-time PCR.

RNA stability analysis. Real-time PCR was performed using cDNA from C. neoformans wild-type H99 cells grown in 2% or 0.06% glucose. Both hnRNA and mRNA were measured, and the ratio of mRNA to hnRNA was calculated as an indicator of mRNA stability (34). Because hnRNA primers contained intronic sequences, the extracted RNA was treated with a DNase kit (Ambion) to ensure that no genomic DNA remained. As a control, to ensure that the DNase treatment removed genomic DNA, no reverse transcriptase was added to RNA samples.
under conditions of low glucose compared to high glucose, this increase was not regulated by temperature or CO₂ concentration (Fig. 4C). Similarly, the App1 protein level increased in low glucose, but it did not increase further when cells were incubated at 37°C and/or 5% CO₂ (Fig. 4D). These results suggest that when \textit{C. neoformans} is exposed to a low-glucose environment, App1 expression is no longer controlled by temperature or CO₂.

**App1 expression is modulated in different body fluids.** We have shown that App1 expression is increased significantly under low-glucose conditions. To determine if App1 expression is modulated throughout the body fluids during infection, human BAL fluid, serum, and CSF samples were obtained and used to incubate \textit{C. neoformans} cells. We first incubated \textit{C. neoformans} cells with BAL fluid, then with serum, and finally with CSF to mimic what would happen during infection in humans (e.g., dissemination from the lungs to the bloodstream and then to the brain). Thus, after 24 h of incubation in BAL fluid, the cells were transferred to serum, and after 24 h in serum, they were transferred to CSF. The incubation temperature was 30°C because we did not find a significant difference in App1 expression between 30°C and 37°C for growth at low glucose concentrations (Fig. 4C and D). After the incubation, cells were subjected to RNA extraction for real-time PCR or to protein extraction for Western blotting. We found a dramatic increase in App1 mRNA expression in BAL fluid compared to App1 mRNA expression in medium containing 2% glucose (Fig. 5A). App1 protein levels were also significantly higher in serum and CSF than in medium with 2% glucose (Fig. 5B). We measured glucose concentrations in human BAL fluid, serum, and CSF and found that glucose in BAL fluid was undetectable, whereas the glucose concentrations in serum and CSF...
were 0.12% (or 120 mg/dl) and 0.075% (or 75 mg/dl), respectively.

A similar experiment was performed with BAL fluid samples obtained from mice, as mice are largely used as an experimental animal model for cryptococcosis. We performed BAL on CBA/J immunocompetent mice (Jackson Laboratory) and Tgε26 immunocompromised mice (MUSC COBRE Animal Core Facility), which lack T and NK cells (45, 46). BAL fluid samples from three mice were pooled and used as is or after the addition of 0.002% glucose.

**FIG. 5.** App1 expression in human body fluids. *C. neoformans* cells were grown in YPD medium with 2% glucose for 24 h, and then cells were transferred to pooled human BAL fluid for 24 h, then to pooled human sera for 24 h, and finally to pooled human CSF for 24 h, all at 30°C. (A) Real-time PCR analysis showing that App1 mRNA expression was increased significantly in BAL fluid, serum, and CSF fluids compared to that in medium with 2% glucose. *, *P < 0.05 for comparison with 2% glucose under the same conditions; §, *P < 0.05 for comparison to 2% glucose at 30°C and 0.04% CO2 by the Student *t* test. Data are averages ± standard deviations for three separate experiments. (B and C) Western blot analysis and quantification of App1 protein expression following cell incubation under various conditions. Data are representative of at least three independent experiments. (D) Glucose concentrations in YPD medium, BAL fluid, serum, and CSF. YPD medium contained 2% glucose, whereas serum and CSF samples contained 0.12% and 0.075% glucose, respectively. Glucose was not detected in BAL fluid samples. Data are averages ± standard deviations for three separate experiments. (B and C) Western blot analysis and quantification of App1 protein expression following cell incubation under various conditions. Data are representative of at least three independent experiments. (D) Glucose concentrations in YPD medium, BAL fluid, serum, and CSF. YPD medium contained 2% glucose, whereas serum and CSF samples contained 0.12% and 0.075% glucose, respectively. Glucose was not detected in BAL fluid samples. Data are averages ± standard deviations for three separate experiments. (E) *C. neoformans* cells were incubated at 30°C for 18 h in mouse BAL fluid recovered from either CBA/J or Tgε26 mice, with or without the addition of 0.002% glucose. App1 expression was increased in mouse BAL fluid, with or without the addition of basal levels of glucose. Data are representative of at least three independent experiments.

**FIG. 4.** App1 expression is upregulated under mammalian physiological conditions in vitro. (A and B) The *C. neoformans* wild-type strain was grown at different temperatures and CO2 levels in medium containing 2% glucose for 18 to 20 h. Western blot analysis (A) and quantification (B) show an increase in App1 expression at 37°C compared to 30°C and at 5% CO2 compared to 0.04% CO2. Data are representative of at least three independent experiments. (C) The *C. neoformans* wild-type (WT) strain was grown at a high (2%) or low (0.06%) glucose concentration for 18 to 20 h at 30°C or 37°C and 0.04% or 5% CO2. Real-time PCR shows that App1 expression was increased in 0.06% compared to 2% glucose under all conditions. In addition, there was a significant increase in App1 expression in high-glucose medium at 37°C and 5% CO2 compared to that under culture conditions of 30°C and 0.04% CO2, *, *P < 0.05 for comparison to 2% glucose under the same conditions; §, *P < 0.05 for comparison to 2% glucose at 30°C and 0.04% CO2 by the Student *t* test. Data are averages ± standard deviations for three separate experiments. (D) The *C. neoformans* wild-type strain was grown at a low (0.06%) glucose concentration for 18 to 20 h at 30°C or 37°C and 0.04% or 5% CO2. Western blot analysis shows App1 expression in 0.06% glucose at 30°C and 0.04% CO2 compared to that at 37°C with 0.04% or 5% CO2.
incubated in the BAL fluid for 18 to 20 h and used for RNA extraction for real-time PCR. As illustrated in Fig. 5E, App1 expression was significantly upregulated in mouse BAL fluid, with or without the addition of a basal level of glucose. We also found that there was no difference in App1 expression in BAL fluid between immunocompetent and immunocompromised mice (Fig. 5E).

**App1 expression is increased in macrophage-conditioned medium.** Since early upon inhalation of *C. neoformans*, several immune cells, including macrophages, are recruited to the site of the infection, we hypothesized that this recruitment would further decrease an already low glucose concentration, with a consequent increase in App1 expression. We sought to test this hypothesis in vitro by using a mouse alveolar macrophage cell line (MHS). Thus, different numbers of MHS cells were incubated in medium with 1% glucose for 72 h. We needed to use a high level of glucose for this experiment to provide the best growing conditions for the cell line and to study the effect of significant glucose consumption by macrophages on App1 expression. Thus, after 72 h of incubation, the medium was centrifuged and only the supernatant was used to incubate *C. neoformans* cells. One set of supernatants was used as is, whereas 1% glucose was added back to a second set before the addition of *C. neoformans* cells. As shown in Fig. 6, App1 expression was upregulated linearly in medium conditioned by increasing numbers of macrophages. Remarkably, in the conditioned medium in which 1% glucose was added back before incubation of fungal cells, the increase in App1 expression was not observed. These results suggest that the increase in App1 expression in macrophage-conditioned medium was due to glucose depletion.

**Both App1 transcription and mRNA stability are increased during glucose starvation.** We next sought to determine which molecular mechanism is responsible for the upregulation of App1. We sought to determine if the increase in App1 expression was due solely to an increase in transcription or if mRNA stability was also affected. Real-time PCR was done using different primer sets to amplify mRNA and hnRNA. Since hnRNA is the direct product of transcription and has not been modified, it is an indicator of the level of transcription (12). In addition, the ratio of mRNA to hnRNA is a means to examine RNA stability which has been used in many previous reports (4, 12, 15, 20, 34, 35). To measure mRNA, primers were used that crossed exon-exon boundaries, detecting only spliced and mature mRNA. To measure hnRNA, primers were used that crossed an exon-intron boundary. Using these primers, only nonspliced naïve RNA would be detected. Interestingly, App1 hnRNA increased under conditions of low glucose, by ~4.7-fold (Fig. 7A), which is significantly less than the App1 mRNA expression illustrated in Fig. 1A (~8-fold). Analysis of the mRNA/hnRNA ratio showed a significant increase at 0.06% glucose compared to 2% glucose (Fig. 7B), suggesting an increase in the mRNA stability of App1 under conditions of low glucose compared to high glucose.

**App1 signaling mechanism during glucose starvation.** Several signaling pathways have been suggested to play a role in sensing the glucose concentration in the environment and thus to adapt fungal cells for the production of virulence factors (e.g., melanin). Thus, we sought to study whether such signal-
Interestingly, we found that 20 h. RNA was then extracted and subjected to real-time PCR. YP medium containing either 2% or 0.06% glucose for 18 to 20 h. Mig1 are proteins involved in the most widely studied glucose repression pathway (19). C. neoformans (28); Snf1, Hxt1, and Pkc1 is in- vestigation from 0.06% to 2% glucose for the two mutants that showed significant decreases in the fold changes of App1 mRNA in low-glucose medium compared to the WT. *P < 0.05 for comparison to WT by the Student t test; §, P < 0.05 for comparison to WT by the Student t test. Data are averages ± standard deviations for three separate experiments. (B) Average fold changes of App1 expression from 0.06% to 2% glucose for the two mutants that showed significant differences in App1 expression compared to the wild type in 2% and/or 0.06% glucose. Both the Δgpa1 and Δsnf1 strains had significant decreases in the fold changes of App1 mRNA in low-glucose medium compared to the WT. *P < 0.05 for comparison to WT by the Student t test. Data are averages ± standard deviations for three separate experiments. (C) The C. neoformans wild-type (WT) strain and Δgpa1 and Δsnf1 mutant strains were grown in 2% or 0.06% glucose for 18 to 20 h. Western blot analysis shows that App1 protein expression increased at 0.06% in the Δgpa1 and Δsnf1 mutants, similar to the increase in WT cells. Data are representative of at least three independent experiments.

FIG. 8. Potential signaling pathways regulating App1 expression during glucose starvation. C. neoformans wild-type (WT or KN99) or mutant strains were grown in 2% or 0.06% glucose for 18 to 20 h. Real-time PCR was performed to measure App1 expression in each mutant at both glucose concentrations. (A) App1 mRNA expression is shown for all mutants at 2% and 0.06% glucose. *P < 0.05 for comparison to WT by the Student t test. Data are averages ± standard deviations for three separate experiments. (B) Average fold changes of App1 expression from 0.06% to 2% glucose for the two mutants that showed significant differences in App1 expression compared to the wild type in 2% and/or 0.06% glucose. Both the Δgpa1 and Δsnf1 strains had significant decreases in the fold changes of App1 mRNA in low-glucose medium compared to the WT. *P < 0.05 for comparison to WT by the Student t test. Data are averages ± standard deviations for three separate experiments. (C) The C. neoformans wild-type (WT) strain and Δgpa1 and Δsnf1 mutant strains were grown in 2% or 0.06% glucose for 18 to 20 h. Western blot analysis shows that App1 protein expression increased at 0.06% in the Δgpa1 and Δsnf1 mutants, similar to the increase in WT cells. Data are representative of at least three independent experiments.

The corresponding mutants for these genes were grown in YP medium containing either 2% or 0.06% glucose for 18 to 20 h. RNA was then extracted and subjected to real-time PCR. Interestingly, we found that Δgpa1 and Δsnf1 mutants had significant increases in App1 expression in 2% glucose, whereas the Δsnf1 strain had a significant decrease in App1 expression in 0.06% glucose compared to the wild-type strain (H99 or KN99) (Fig. 8A and B). Interestingly, the fold changes of App1 expression under conditions of low glucose compared to high glucose for both Δsnf1 and Δgpa1 mutants were significantly lower than those observed for wild-type cells, suggesting that Snf1 and/or Gpa1 protein may play a role in activating App1 transcription. However, when we examined the level of App1 protein under conditions of low versus high glucose, we found no significant change in both Δsnf1 and Δgpa1 mutants compared to the wild type (Fig. 8C).

**DISCUSSION**

Studies of how fungi sense the environment have significantly increased in the last decade. Regarding the pathogenic fungus C. neoformans, several signaling pathways have been identified to respond to different environmental stresses, such as high temperature, high/low CO2 concentrations, and nutrient deprivation (3). Here we report that C. neoformans App1 is significantly upregulated when cells are starved of glucose (low-glucose medium) or exposed to body fluids, such as BAL fluid, serum, or CSF. We also found that upregulation of App1 is not controlled by other signaling pathways that are upregulated during glucose starvation.

App1 expression was significantly upregulated when fungal cells were exposed to low glucose concentrations (Fig. 1 and 2). Interestingly, this also occurred when C. neoformans cells were exposed to low galactose concentrations (Fig. 3). Interestingly, the magnitudes of changes in App1 expression were greater in the presence of certain concentrations of galactose versus glucose (compare Fig. 3 with Fig. 2). Recent studies with Candida albicans have shown that many genes upregulated during glucose starvation are also upregulated during galactose starvation (7). In particular, C. albicans senses both sugars through Hgt4, which then leads to phosphorylation of Rgt1, resulting in the transcription of various genes (40). Our results suggest that the pathway leading to App1 upregulation during glucose starvation may be more similar to that in C. albicans than to that in S. cerevisiae, which responds to glucose and galactose through different pathways (5, 7). Both HGT4 and RGT1 genes seem to be present in the C. neoformans genome (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html), and the C. albicans sugar transporter Hgt4 and transcription factor Rgt1 genes are highly similar to several uncharacterized C. neoformans transcripts. No hits were found when the H99 genome was BLAST searched using the S. cerevisiae RGT1 sequence. Determination of the roles of the C. neoformans homologs for C. albicans HGT4 and RGT1 in the activation of App1 upon glucose starvation awaits further studies.

C. neoformans is an environmental fungus found mostly in pigeon droppings, in which glucose concentrations can reach up to 0.1% of dry feces weight (31). Other species of Cryptococcus, such as Cryptococcus gattii, are associated mostly with soil and trees, in which glucose concentrations vary significantly: the glucose concentration can reach up to 0.5% in dry soil (47), and it can vary from 0.1 to 0.4% in soil (47), and it can vary from 0.1 to 0.4% in soil (40). Thus, when inhaled into the lung, these species can experience glucose starvation (3). Here we report that C. neoformans App1 expression increases in response to glucose deprivation (3). Here we report that C. neoformans App1 expression increases in response to glucose deprivation (3).
(or higher) to 0.002%, supporting the hypothesis that when *C. neoformans* or *C. gattii* is inhaled into the alveolar spaces, App1 will be upregulated.

We also found a direct effect of glucose concentration on App1 expression: the higher the glucose concentration, the lower the App1 expression level. Upregulation of App1 is quite rapid, and by 16 to 24 h of incubation of fungal cells in low-glucose medium, both App1 mRNA and protein are significantly increased. Thus, it is reasonable to hypothesize that increases in App1 in inhaled fungi will occur within hours or 1 to 2 days after infection. Upregulation of App1 in the lung appears not to be controlled by high concentrations of CO2 or high (37°C) temperatures, because exposure to these environments under glucose starvation conditions did not further increase App1 expression (Fig. 4C and D).

Because App1 expression *in vitro* correlates with the glucose concentration, we hypothesized that App1 expression in human body fluids is also regulated by their low glucose content. We incubated *C. neoformans* cells in BAL fluid, then in serum, and finally in CSF to mimic the route of the fungus upon inhalation (from the lungs to the bloodstream and then to the brain). App1 mRNA was highly expressed in BAL fluid and significantly increased, although to a lesser degree, in serum and CSF compared to that in high-glucose medium (Fig. 5A). Similarly, App1 protein increased in BAL fluid but not in serum or CSF (Fig. 5B and C), although it is possible that serum and/or CSF contains a factor(s) that inhibits App1 protein production and/or enhances App1 protein degradation. Nevertheless, our results clearly show that the lung is the primary site at which both App1 mRNA and protein are upregulated.

During the early stage postinhalation in the lung, *C. neoformans* cells encounter mostly resident alveolar macrophages, which have yet to be activated (32, 33). When we studied the expression of App1 in cells exposed to medium collected from a nonactivated macrophage cell line, we found that App1 significantly increased with the number of macrophages used to condition the medium (Fig. 6, conditioned medium) and did not change when glucose was added back to the conditioned medium (Fig. 6, conditioned medium + 1% glucose). These results suggest that the presence of macrophages will most likely affect App1 production by *C. neoformans* cells through the consumption of glucose in the surrounding extracellular space.

In previous studies, we found that the virulence of a mutant lacking App1 (Δapp1) is different in an immunocompetent versus an immunocompromised (Tg2) (26) mouse model which lacks both T and NK cells (45, 46). In immunocompetent mice, the Δapp1 mutant is less virulent, whereas in Tg26 mice the Δapp1 mutant is hypervirulent compared to the *C. neoformans* wild-type strain, and the *C. neoformans* wild-type strain is more virulent in immunocompromised than immunocompetent mice (26). Thus, we investigated whether App1 expression would differ in *C. neoformans* cells exposed to BAL fluid isolated from immunocompetent versus immunocompromised mice. Our results showed that App1 expression is not regulated by the immune status of the host (at least in BAL fluid) but rather by the glucose concentration in this environment (Fig. 5E), further suggesting the importance of deprivation of this nutrient for App1 at the initial site of the infection.

Upregulation of App1 under conditions of low glucose is regulated by both an increase in transcription and an increase in mRNA stability. We first tested whether App1 expression would be regulated by the Ipc1-DAG-Atf2 pathway by measuring luciferase activity and the Atf2 protein level in an *IPC1/app1::LUC/ATF2::HA* strain, in which the luciferase gene (LUC) is under the control of the *APP1* promoter and the *ATF2* gene is tagged with the hemagglutinin (HA) epitope (43). We found that neither luciferase activity nor Atf2 protein was increased when the cells were exposed to low glucose compared to high glucose concentrations (data not shown). Although these results do not exclude a possible role for Atf2 in the regulation of App1 mRNA stability, they suggest that App1 transcript is not regulated by Atf2 under low-glucose conditions. It is possible that a different transcription factor may be involved in App1 regulation at low glucose concentrations. Indeed, in previous studies, we found that in addition to deletion of the ATF cis-acting element, deletion of the AP-2 sequence in the *APP1* promoter abrogated App1 activation by DAG (43), suggesting that DAG may also regulate App1 through a signaling pathway different from the Atf2 pathway.

Because DAG activates Pkc1 in *C. neoformans*, we next investigated whether App1 expression is regulated by the Ipc1-DAG-Pkc1 pathway, based on previous observations in which inhibition of Pkc1 activity by treatment with calphostin C decreased *APP1* gene transcription in high-glucose medium (28). Thus, we measured App1 mRNA in a *C. neoformans* strain in which the C1 domain of Pkc1 was deleted (ΔC1-Pkc1) and Pkc1 activity was no longer controlled by Ipc1 and DAG. We found that App1 mRNA in the ΔC1-Pkc1 strain was still upregulated under conditions of low glucose compared to high glucose, although this occurred to a lesser extent compared to the case for wild-type cells (Fig. 8). These results suggest that Pkc1 is not involved in the regulation of App1 mRNA under low-glucose conditions.

Finally, we examined all pathways in *C. neoformans* identified thus far to be controlled by glucose starvation and found that they do not regulate App1 expression (at least at the protein level). Indeed, App1 expression in Δgpa1, Δsnf1, Δsnf5, Δmig1, and ΔSkt1 mutants was found to be upregulated at low glucose concentrations, similar to that in *C. neoformans* wild-type cells (Fig. 8). Interestingly, upregulation of App1 mRNA under conditions of low glucose was impaired in the Δgpa1 and Δsnf1 mutants (Fig. 8A and B), but this impairment was not confirmed at the protein level (Fig. 8C), suggesting that these pathways may have some control in App1 gene transcription or mRNA stability.

In conclusion, in this study, we found that App1 is dramatically upregulated in *C. neoformans* cells exposed to a low-glucose environment. This regulation appears to affect App1 at both the transcriptional and posttranscriptional levels and is not controlled by signaling pathways identified thus far to be activated by glucose starvation.

**ACKNOWLEDGMENTS**

We thank all members of the Del Poeta laboratory for comments. We are particularly grateful to Chiara Luberto for helpful and constructive discussions.

This work was supported by R01 grants AI56168 and AI72142 (to M.D.P.) from the National Institutes of Health and was conducted in a facility constructed with support from the National Institutes of Health.
REFERENCES

20. Larente, R. C., M. A. Treitel, and M. Carlson. 1990. The SNF5 protein of Saccharomyces cerevisiae is a glutamine-and-proline-rich transcriptional ac-