Cryptococcus neoformans mating and virulence are regulated by the G-protein α subunit GPA1 and cAMP

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This study explores signal transduction pathways that function during mating and infection in the opportunistic, human fungal pathogen Cryptococcus neoformans. The gene encoding a G-protein α subunit homolog, GPA1, was disrupted by homologous recombination. The gpa1 mutant strain was viable but exhibited a defect in mating in response to nitrogen starvation. Additionally, the gpa1 mutant strain failed to induce two well-established virulence factors—melanin synthesis, in response to glucose starvation; and capsule production, in response to iron limitation. As a consequence, virulence of the gpa1 mutant strain was significantly attenuated in an animal model of cryptococcal meningitis. Reintroduction of the wild-type GPA1 gene complemented the gpa1 mutant phenotypes and restored mating, melanin and capsule production, and virulence. Similarly, exogenous cAMP also suppressed the gpa1 mutant phenotypes, restoring mating and production of melanin and capsule. These observations support a model in which GPA1 has a role in sensing diverse environmental signals required for mating and virulence by regulating cAMP metabolism in C. neoformans.

[Key Words: Cryptococcus; G protein; signal transduction; pathogenesis; capsule; melanin]

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All cells must be able to sense and respond to changes in the environment. In the specific case of pathogenic microorganisms, a dramatic change in external signals occurs as the organism enters the host. There, host-specific signals often induce the production of microbial virulence factors that allow the pathogen to survive within this new environment (Mekalanos 1992). Elements of these signaling and response pathways in microorganisms include cell-surface receptors, adapter proteins such as guanine nucleotide-binding proteins (G proteins) or protein kinases, and regulatory elements including second messengers and transcriptional regulators. Our studies focus on how signal transduction cascades regulate virulence in pathogenic fungi.

Cryptococcus neoformans is a heterothallic, basidiomycetous, pathogenic fungus that most often infects immunocompromised patients. The prevalence of disease caused by this organism has increased dramatically as a result of human immunodeficiency virus (HIV) infection, organ transplantation, cytotoxic chemotherapy, and corticosteroid use.

C. neoformans is one of the most common invasive, opportunistic pathogens in acquired immune deficiency syndrome (AIDS) patients and is the most common cause of fungal meningitis worldwide (Mitchell and Perfect 1995). Established pathogenic traits of this organism include an antiphagocytic polysaccharide capsule (Granger et al. 1985; Kwon-Chung and Rhodes 1986; Chang et al. 1996), melanin elaborated within the cell wall as an antioxidant (Kwon-Chung et al. 1982; Wang et al. 1995; Salas et al. 1996), the ability to grow at 37°C (Kwon-Chung and Rhodes 1986; Odom et al. 1997), prototrophy (Perfect et al. 1993), and α-mating type (Kwon-Chung et al. 1992).

Environmental signals that induce virulence factors in C. neoformans have been identified previously. For example, capsule formation in C. neoformans is induced by severe iron deprivation (Vartivarian et al. 1993) or exposure to mammalian physiologic CO₂/HCO₃⁻ levels (Granger et al. 1985). Melanin production requires the presence of diphenolic substrates and is induced by glucose deprivation (N urudeen and Ahearn 1979; Polacheck et al. 1982; Torres-Guererro and Edman 1994; Williamson 1994). Similarly, mating and haploid fruiting, both of which may serve to produce small (1–2µ), potentially infectious spores, are induced by nitrogen starvation (Kwon-Chung 1976; Wickes et al. 1996). Although some of the environmental signals that induce virulence factors have been identified, the specific signal transduction cascades that detect these signals and regulate expression of these and other virulence factors have only recently begun to be elucidated (Odom et al. 1997).
Recently, several virulence-associated signaling cascades in plant fungal pathogens have been found to involve regulation by G proteins. For example, the Gα subunit homolog Gpa3 is required for mating and virulence in the maize pathogen Ustilago maydis (Regenfelder et al. 1996). Similarly, the Gα subunit Cpg-1 regulates mating and virulence in Cryphonectria parasitica, the cause of chestnut blight (Gao and Nuss 1996). A gene encoding a cryptococcal G-protein α-subunit, GPA1, was identified previously (Tolkacheva et al. 1994). By analogy with the roles in mating of the GPA1 and Gpa1 Gα subunits in budding and fission yeasts, it was hypothesized that the C. neoformans GPA1 protein might be coupled to a pheromone receptor and therefore also have a role in mating. However, by sequence homology comparisons, we find that GPA1 shares greater identity with a distinct class of Gα proteins involved in sensing nutrient deprivation during pseudohyphal growth in Saccharomyces cerevisiae (GPA2) (Lorenz and Heitman 1997) and mating in Schizosaccharomyces pombe (Gpa2) (Nakafuku et al. 1988; Ishiiki et al. 1992; Regenfelder et al. 1996). Because several of the established pathogenic factors in C. neoformans are induced by nutrient starvation, we hypothesized that GPA1 might regulate nutrient signaling and virulence in this organism.

To test this hypothesis, we disrupted the C. neoformans gene encoding the G-protein α subunit homolog GPA1. In contrast to the isogenic GPA1 wild-type strain, the gpa1 mutant strain exhibited a severe mating defect. Importantly, the gpa1 mutant strain also failed to induce two well-established virulence factors, capsule and melanin, in response to environmental signals. Correspondingly, the gpa1 mutant strain was significantly less virulent than the isogenic GPA1 mutant strain by transforming the gpa1 + GPA1 wild-type strain, in which a 1.3-kb GPA1 fragment containing the endogenous GPA1 allele and the wild-type GPA1 gene were now present, as indicated by PCR amplification of both a 4.0-kb (gpa1::ADE2) and a 1.6-kb (GPA1) DNA fragment (Fig. 1C) and Southern blot analysis (data not shown). This transformant was denoted as the gpa1 + GPA1 strain.

Reintroduction of the GPA1 gene in the gpa1 mutant strain

The wild-type GPA1 gene was reintroduced into the gpa1 mutant strain by transforming the GPA1 gene linked to a fusion gene conferring hygromycin B resistance (Cox et al. 1996). Genomic DNA was isolated from five hygromycin B-resistant transformants and analyzed by PCR with primers flanking the GPA1 locus. In one of the five transformants, both the gpa1::ADE2 mutant allele and the wild-type GPA1 gene were now present, as indicated by PCR amplification of both a 4.0-kb (gpa1::ADE2) and a 1.6-kb (GPA1) DNA fragment (Fig. 1C) and Southern blot analysis (data not shown). This transformant was denoted as the gpa1 + GPA1 strain.

To examine expression of the GPA1 gene, Northern analysis was performed on total RNA isolated from the isogenic GPA1 wild-type parental strain, the gpa1::ADE2 mutant, and the gpa1 + GPA1 reconstituted strain. In contrast to the GPA1 wild-type strain, in which a 1.3-kb message was readily detectable, no message was detected in the gpa1 mutant strain, even when grown in nitrogen-limited medium (Fig. 1D). This observation supports the conclusion that GPA1 has been functionally deleted in the gpa1::ADE2 mutant strain. Following reintroduction of the wild-type GPA1 gene by transformation, GPA1 expression was restored in the gpa1 + GPA1 strain. The more abundant GPA1 message detected in the gpa1 + GPA1 strain may be indicative of multiple functional copies of the GPA1 gene introduced ectopically by the transformation procedure.

The wild-type, gpa1, and gpa1 + GPA1 strains displayed identical growth rates in both liquid culture and on solid media at 25, 30, and 37°C. In addition, all three
strains grew on minimal YNB media, indicating that mutation of \( \text{GPA1} \) does not confer any novel auxotrophies (data not shown).

**GPA1** is required for mating in *C. neoformans*

When cocultured on solid media limiting for nitrogen, cryptococcal cells of opposite mating type mate, undergoing a filamentous morphological change that ultimately results in nuclear fusion, meiosis, and sporulation (Kwon-Chung 1976). In contrast, when one or more of the mating partners is sterile, no filaments or spores are formed from the mating mixtures. Filament and spore formation in response to a mating partner under appropriate conditions therefore serves as an assay for mating in *C. neoformans*.

We tested whether the GPA1 gene is required for mating in *C. neoformans*. The wild-type GPA1 \( \text{MAT}^\alpha \), serotype A strain (H99) produced prominent hyphae and abundant basidiospores within 7 days when crossed with a serotype D strain of opposite mating type (JEC20, \( \text{MAT}^\alpha \)). In contrast, only scarce and rudimentary filaments formed when the \( \text{gpa1} \) mutant \( \text{MAT}^\alpha \) strain was crossed with the \( \text{MAT}^\alpha \) mating partner JEC20 (Fig. 2). Reintroduction of the GPA1 gene complemented this mating defect, restoring filament and basidiospore production in the \( \text{gpa1} \) mutant strain to the extent observed with the wild-type GPA1 strain (Fig. 2). Note that for these crosses a serotype D mating partner was used because no serotype A, \( \text{MAT}^\alpha \) isolate has yet been identified (Kwon-Chung and Bennett 1978).

**GPA1** regulates capsule production in *C. neoformans*

As a means to test whether GPA1 is involved in sensing...
pheromones or more general environmental signals, we tested the effects of the gpa1::ADE2 mutation on the induction of virulence factors in response to nutrient deprivation. Visual inspection of the GPA1, gpa1, and gpa1 + GPA1 strains on different solid media revealed that the gpa1 mutant strain forms very dry colonies compared with the mucoid colonies formed by the wild-type GPA1 and gpa1 + GPA1 strains. This difference in colony morphology is attributable to a marked decrease in capsule production by the gpa1 mutant, even under capsule-inducing conditions. When grown in liquid media under extreme iron limitation (low iron media + 56 µM ethylenediamine-di(o-hydroxy-phenylacetic acid) [LIM + EDDHA] (Vartivarian et al. 1993), capsule production is induced in the GPA1 wild-type and gpa1 + GPA1 strains and was observed readily when the cells are examined by a standard India ink preparation (Fig. 3). In contrast, gpa1 mutant cells grown under the same conditions exhibited little or no capsule when stained by the same technique (Fig. 3).

Capsule size and cell volume have been qualified previously by measuring the packed cell volume of suspensions of C. neoformans cells (termed the cryptocrit, by analogy with hematocrit) (Granger et al. 1985). The GPA1 wild-type, gpa1 mutant, and gpa1 + GPA1 strains were grown in low iron media (LIM + EDDHA) for 48 hr, pelleted, and resuspended at 10⁶ cells/ml after treatment with 10% formalin. The packed cell volume for the suspensions was 34% for the wild-type GPA1, 5.4% for the gpa1 mutant, and 29% for the gpa1 + GPA1 strains. Therefore, by this quantitative measurement, the capsule size was also reduced dramatically in the gpa1 mutant strain compared with the GPA1 wild-type strain or the gpa1 + GPA1 complemented strain, confirming the macroscopic and microscopic observations on the difference in capsule size among the three strains.

GPA1 regulates melanin production in C. neoformans

C. neoformans produces melanin within the cell wall when grown in the presence of diphenolic compounds that serve as substrates for melanin production by phenoloxidase (Nurudeen and Ahearn 1979). In addition, glucose deprivation serves as an environmental signal that induces melanin production. When cultured on Ni-

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**Figure 2.** GPA1 regulates mating in C. neoformans. The isogenic GPA1 wild-type, gpa1 mutant, and gpa1 + GPA1 MATa strains were co-incubated with a MATa mating partner (strain JEC20) on nitrogen limiting mating media (V8 agar) for 7 days at 25°C. The edges of the mating mixtures were photographed (50×).

**Figure 3.** GPA1 regulates capsule formation in C. neoformans. The isogenic GPA1 wild-type, gpa1 mutant, and gpa1 + GPA1 strains were cultured for 48 hr at 30°C in liquid low iron media with 56 µM EDDHA (an iron chelator). The polysaccharide capsule was stained by a standard India ink preparation, and the cells were photographed (200×).
The rate-limiting step in melanin production is catalyzed by the enzyme phenoloxidase. We wished to address whether the phenoloxidase enzyme is expressed and functional in the gpa1 mutant cells. For this purpose, we employed a quantitative assay involving spectrophotometric measurement of melanin production by permeabilized cells exposed to diphenolic compounds (Polacheck et al. 1982; Rhodes 1986; Torres-Guererro and Edman 1994). Cultures of the GPA1 wild-type, gpa1 mutant, and gpa1 + GPA1 reconstituted strains were grown in glucose-deprivation media and permeabilized with toluene:ethanol. Caffeic acid was added to the cell suspensions, and the absorbance monitored at 480 nm. By this assay, no melanin was produced by the permeabilized gpa1 mutant cells, whereas significant amounts were produced by the GPA1 wild-type and the gpa1 + GPA1 complemented strains (Fig. 4B). These findings suggest that the gpa1 mutant strain fails to express active phenoloxidase enzyme under glucose starvation conditions, especially when compared with the GPA1 wild-type and gpa1 + GPA1 strains.

GPA1 is required for growth of C. neoformans in vivo
Cryptococcal mutants deficient in melanin or capsule production are less virulent than wild-type strains in animal models of cryptococcal infection (Fromtling et al. 1982; Kwon-Chung et al. 1982; Torres-Guererro and Edman 1994; Chang et al. 1996). Because GPA1 is required to induce capsule and melanin production in response to environmental signals in vitro, we tested whether GPA1 is required for virulence in vivo. To address this issue, we assessed the ability of the GPA1, gpa1, and gpa1 + GPA1 strains to survive in the maintenance phase of a meningeal infection using a rabbit model of cryptococcal meningitis (Perfect et al. 1980). Corticosteroid-immunosuppressed rabbits were inoculated intrathecaly with equivalent numbers of cells of each of the three strains, and quantitative cerebrospinal fluid (CSF) cultures were obtained over the course of a 2-week infection. By the 10th day of infection, there was a >10,000-fold difference between the number of gpa1 mutant cells remaining in the CSF of infected rabbits compared with the isogenic GPA1 wild-type strain (Fig. 5).

Reintroduction of the wild-type GPA1 gene (gpa1 + GPA1 strain) largely, albeit not completely, complemented this gpa1 mutant phenotype to restore virulence (Fig. 5). The wild-type level of virulence was not completely recapitulated in this reconstituted strain, which may be attributable to differences in expression between the endogenous and ectopic GPA1 genes.

Exogenous cAMP suppresses gpa1 mutant phenotypes
In other microorganisms, Gα subunits with identity to the C. neoformans GPA1 protein regulate cAMP production via adenylate cyclase. We therefore tested whether GPA1 has an analogous role in this organism by testing

Figure 4. GPA1 regulates melanin production in C. neoformans. (A) The GPA1 wild-type, gpa1 mutant, and gpa1 + GPA1 strains were grown on Niger seed agar for 7 days at 37°C. Strains that produce melanin (GPA1, gpa1 + GPA1) are brown on this media, whereas strains that do not produce melanin (gpa1 mutant) are white. (B) The GPA1 wild-type, gpa1 mutant, and gpa1 + GPA1 strains were grown for 16 hr at 37°C with glucose starvation (as described in Materials and Methods). Cells were permeabilized with toluene:ethanol, incubated for 2 hr in the presence of the diphenolic substrate caffeic acid, and cellular phenoloxidase activity assayed spectrophotometrically by measuring the appearance of melanin in the supernatant by the change in absorbance at 480 nm. Values represent the mean of three separate but identical cultures for each strain; error bars indicate standard error of the mean.
whether cAMP could suppress any or all of the gpa1 mutant phenotypes.

The mating defect of the gpa1 mutant was suppressed by exogenous cAMP. When 10 mM cAMP was added to V8 (nitrogen-poor) mating media, mating filaments were observed by 48 hr around the mating mixture of gpa1 mutant cells and a MATa mating partner, JEC20 (data not shown). Following 7–10 days of incubation, abundant filaments and basidiospores were present (Fig. 6). cAMP did not significantly enhance mating by either the GPA1 wild-type or gpa1 + GPA1 strains, nor did it allow any of the three strains to mate under nitrogen-rich conditions (data not shown). Additionally, cAMP did not stimulate haploid fruiting by the GPA1, gpa1, or gpa1 + GPA1 strains grown on V8 media over the course of a 2 week period (data not shown).

In addition, 10 mM cAMP in either solid or liquid media restored capsule formation in the gpa1 mutant and further induced capsule in the GPA1 wild-type strain grown in capsule-inducing media (Fig. 7A, B). Most striking was the vigorous production of a polysaccharide capsule by the otherwise hypocapsular gpa1 mutant cells in cAMP-containing, low-iron media. The gpa1 mutant cells, which have a slightly smaller diameter than their GPA1 wild-type counterparts when grown in the absence of exogenous cAMP, were indistinguishable from wild-type when 10 mM cAMP was added to the media (Fig. 7A, B). Interestingly, wild-type cryptococcal cells were stimulated to produce an even larger capsule by cAMP in iron-limiting conditions. Addition of cAMP to iron-rich media failed to induce either the GPA1 wild-type or gpa1 mutant strain to produce capsule, indicating that the iron-deprivation signal is still required for this induction (Fig. 7A, B).

Similarly, exogenous cAMP also suppressed a third gpa1 mutant phenotype and restored the ability to produce melanin. The addition of cAMP to melanin-inducing, low-glucose media stimulated production of wild-type levels of melanin in the gpa1 mutant strain. The GPA1 wild-type, gpa1 mutant, and gpa1 + GPA1 reconstituted strains produced equivalent amounts of melanin.
on Niger seed agar media containing 10 mM cAMP (Fig. 7C).

Discussion

Many signal transduction pathway components are remarkably conserved from organisms as diverse as yeast and fungi to man. Elucidating these remarkably conserved processes is fundamental to our understanding of basic physiological events common to all cells. Whereas components of these signaling cascades are often highly conserved, in many cases elements have been co-opted to perform different functions in different species (Blumer and Johnson 1994; Gilman 1995; Levin and Errede 1995). Therefore, for example, G proteins are highly conserved signaling components, yet function in pathways that detect an array of ligands including photons, pheromones, nutrients, and neurotransmitters (Gilman 1995). Our studies have addressed the signal transduction cascades regulating virulence of pathogenic fungi, in part because the signaling cascades that regulate expression of virulence factors in microorganisms are attractive targets for antimicrobial drug development.

GPA1 regulates mating and virulence in C. neoformans

A gene encoding a G protein a subunit homolog was identified previously in C. neoformans and hypothesized to have a role in mating, analogous to the GPA1 and Gpa1 proteins of S. cerevisiae and S. pombe that function in detecting pheromone (Tolkacheva et al. 1994). By sequence comparisons, however, we found that the C. neoformans GPA1 protein shares greater identity with a different class of G a subunits that signal nutrient starvation signals during mating and differentiation in budding and fission yeast. We took a gene disruption approach to test the hypothesis that the C. neoformans GPA1 protein has an analogous role in sensing nutrient deprivation signals in this important fungal pathogen.

C. neoformans gpa1 mutant strains were obtained by
homologous recombination following transformation with a gpa1::ADE2 disruption allele. The resulting gpa1 mutant cells were viable, but exhibited a severe defect in mating, consistent with the hypothesis that GPA1 has a role in mating in this organism. These findings, however, did not distinguish whether GPA1 functions to sense pheromone or nitrogen starvation, both of which are required for mating in this organism. To address this issue, we tested whether GPA1 has a role in sensing other nutrient deprivation signals.

Our findings reveal that the GPA1 Gα subunit homolog has a critical role in regulating virulence of C. neoformans. Importantly, the gpa1 mutant cells failed to produce two well-established virulence factors, melanin and capsule, in response to environmental nutritional starvation signals. Melanin is thought to provide cellular protection from those reactive oxygen and nitrogen species produced by immune cells (Wang et al. 1995), and the polysaccharide capsule has been shown to inhibit phagocytosis (Granger et al. 1985). Mutants deficient in either of these two factors have marked reductions in virulence in animal models of cryptococcosis (Fromtling et al. 1982; Kwon-Chung et al. 1982; Chang and Kwon-Chung 1994; Torres-Guererro and Edman 1994). Correspondingly, the gpa1 mutant strain was avirulent in an animal model of cryptococcal meningitis. Reintroduction of the wild-type GPA1 gene complemented the gpa1 mutant phenotypes. These observations reveal a role for GPA1 in regulating signaling cascades required for a variety of different physiological responses beyond mating, and are consistent with the hypothesis that GPA1 senses the nutrient deprivation signals common to these differentiation events. These observations provide evidence that GPA1 is conserved, both in sequence and in function, with the nutrient-sensing Gα proteins of S. cerevisiae (GPA2) and S. pombe (Gpa2).

Gα subunits have conserved signaling roles in yeast and fungi

In several yeasts and other fungi, Gα proteins have been implicated in signal transduction pathways governing mating, filamentous growth, and virulence. For example, in the basidiomycete U. maydis, the causative agent of corn smut, four genes encoding Gα proteins have been identified. One of these Gα subunits, Gpa3, is required for both virulence and mating (Regenfelder et al. 1996). Similarly, in C. parasitica, an ascomycetous plant pathogen that causes chestnut blight, a G-protein-linked signal transduction pathway also regulates virulence. A hypovirus that infects this fungal pathogen attenuates virulence by inhibiting the Gα protein CPG-1 (Choi et al. 1995). Disruption of the CPG-1 gene results in multiple additional phenotypes, including defects in mating, asexual sporulation and, most interestingly, melanin production (Gao and Nuss 1996).

In both S. cerevisiae and S. pombe, heterotrimeric G proteins are involved in the mating response by signaling the presence of pheromone (Whiteway et al. 1989; Obara et al. 1991). Both of these organisms, however, possess a second Gα protein responsible for signaling nutritional deprivation (Nakafuku et al. 1988; Isshiki et al. 1992; Lorenz and Heitman 1997). In S. pombe, as in C. neoformans, two signals are required for mating—pheromone and nitrogen starvation. Most interestingly, in S. pombe, the Gpa1 Gα protein signals pheromone presence, whereas a second Gα protein, Gpa2, transduces the nitrogen starvation signal. Mutation of the S. pombe gpa2 gene impairs signaling of nutrient deprivation conditions; gpa2 null mutants mate under nitrogen-rich conditions, whereas dominant active Gpa2 alleles result in a partial sterile phenotype (Isshiki et al. 1992). Similarly, the S. cerevisiae GPA2 Gα protein has an analogous signaling role during pseudohyphal differentiation in response to nitrogen starvation (Lorenz and Heitman 1997).

G-protein regulation of cAMP-dependent pathways in C. neoformans and other fungi

We also found that the gpa1 mutant phenotypes could be suppressed by exogenous cAMP, restoring mating, and production of both melanin and capsule in response to environmental signals. These observations provide evidence that one of the cellular functions of GPA1 may be to regulate cAMP-dependent pathways in C. neoformans. Strikingly, similar hypotheses have emerged recently for signaling cascades regulating pseudohyphal differentiation in the yeast S. cerevisiae (Lorenz and Heitman 1997), mating in S. pombe (Isshiki et al. 1992), and mating and virulence in the plant fungal pathogens U. maydis (Gold et al. 1994) and C. parasitica (Chen et al. 1996). In these diverse organisms, highly conserved Gα subunits regulate cAMP-dependent cellular differentiation pathways.

In S. cerevisiae, it has been shown recently that cAMP stimulates pseudohyphal differentiation and suppresses the pseudohyphal defect of mutants lacking the GPA2 Gα subunit (Lorenz and Heitman 1997). GPA2 had been implicated previously in regulating cAMP production in yeast (Nakafuku et al. 1988; Papasavvas et al. 1992). In S. pombe, gpa2 null mutants mate even in the presence of high levels of nitrogen and have cAMP levels that are reduced to one-third the level of Gpa2 wild-type cells. In both S. cerevisiae and S. pombe, gpa2 mutant cells fail to increase cAMP levels in response to glucose stimulation (Nakafuku et al. 1988; Isshiki et al. 1992). These studies reveal a diverse role for Gα proteins in the regulation of cAMP metabolism and differentiation.

cAMP has also been implicated in signaling cascades that regulate filamentous growth and virulence of plant fungal pathogens. In the dimorphic pathogenic fungus U. maydis, deletion of the gene encoding adenylate cyclase, Uac1, results in a constitutive filamentous phenotype. The addition of exogenous cAMP to uac1 mutants restores a normal budding morphology (Gold et al. 1994). Analogous to our findings in C. neoformans, a G-protein pathway regulating virulence in the chestnut blight fungus C. parasitica also involves regulation of cAMP. In this case, however, the CPG1 protein serves to decrease...
cAMP levels, which results in increased virulence (Chen et al. 1996). Our findings suggest that cryptococcal GPA1 stimulates cAMP production, resulting in increased melanin, capsule formation, and virulence. Although in some cases conserved Gα proteins activate, whereas others inhibit cAMP production, taken together, these findings support a conserved role for Ga subunits in regulating cAMP-dependent signaling pathways regulating mating, filamentous growth, and virulence in yeast and fungi.

Implications

Based on our findings, we propose a model outlining the mechanisms by which external signals regulate the expression of virulence factors in C. neoformans (Fig. 8). Our studies reveal that the Gα protein homolog GPA1 has a central role in regulating mating, induction of virulence factors, and virulence in C. neoformans. Our hypothesis is that GPA1 is one component of a signal transduction cascade that senses diverse environmental nutritional starvation signals and appropriately regulates cell differentiation events. The simplest hypothesis consistent with our findings is that GPA1 activates adenylate cyclase to produce cAMP, a role similar to that of related Gα subunits in other fungi and multicellular eukaryotes (Gilman 1995). An alternative hypothesis that cannot be formally excluded is that cAMP lies in a parallel signaling cascade that, when activated, can bypass the need for GPA1 function. Given that cAMP fully suppressed all of the gpa1 mutant phenotypes, and that cAMP clearly stimulates capsule production in GPA1 wild-type cells and therefore can function in signaling pathways regulating the acquisition of virulence, we favor the hypothesis that GPA1 regulates cAMP metabolism.

Different environmental signals regulate mating, melanin production, and capsule production in C. neoformans. GPA1 serves as a signaling component common to all of these pathways, yet there is little or no cross-induction of these responses by the other environmental signals transduced via GPA1. For example, capsule is induced in response to iron limitation but not in response to glucose or nitrogen deprivation. Similarly, mating occurs in response to nitrogen starvation but not in response to glucose or iron limitation. The only example of cross induction that we have observed involves some modest melanin production in response to nitrogen or iron limitation when diphenolic substrates are present (data not shown).

Although GPA1 is involved in regulating mating and the expression of several virulence traits in C. neoformans, it is clearly not the only component of these signaling cascades, as distinct environmental signals give rise to different cellular responses. Notably, the specific cell surface or intracellular receptors that initiate these signaling cascades remain to be identified. At present, it is also not clear whether GPA1 is a subunit of a heterotrimeric G protein or acts as a solo α-subunit, because no Gβ or Gγ subunits have as yet been identified in this organism. Therefore, additional components of the signaling cascades regulating virulence remain to be identified.

In conclusion, our studies and related investigations reveal that Gα subunits fulfill multiple cellular signaling functions in diverse yeast and fungi. Moreover, these studies reveal that some signaling components are common to pathways regulating both virulence and other cellular processes, such as mating. These findings suggest that signaling pathways that originally evolved to enable organisms to respond to nutritional starvation in the environment may have been co-opted during the evolution of pathogenic organisms to serve similar regulatory functions in the often harsh environment of the infected host.

Materials and methods

Strains and media

C. neoformans strains used were derivatives of the serotype A, MATα strain H99, including the ade2 mutant M049. Mating assays with these strains were performed with the serotype D, MATa strain JEC20 (Moore and Edman 1993). Strains were grown routinely and maintained on rich medium (YPD) unless otherwise stated, and auxotrophic mutations were assessed on synthetic media and yeast nitrogen base (YNB) medium used routinely for S. cerevisiae (Sherman 1991). Mating assays were performed on V8 mating medium containing 5% V8 juice (Campbell's Soup Co.), 0.5 gram/liter of KH2PO4, and 4% Bactoagari (Difco) with the pH adjusted to 7.2 before autoclaving. Regeneration medium for biolistic transformations was as described previously (Toffaletti et al. 1993). Melanin production was assessed on Niger seed (Guizotia abyssinica) medium containing extract of 70 grams of Niger seed per liter (Niger seed
pulverized and boiled for 15 min, then filtered through cheese-cloth) and 4% Bacto-agar. Capsule production was assessed in low iron medium plus 56 µM ethylenediaminedi(o-hydroxyphenylacetic acid) (LIM + EDDHA) as described previously (Var-tiervarian et al. 1993). Synthetic low ammonia media (SLAD) contains 1.7 gram/liter of yeast nitrogen base without amino acids and ammonium sulfate (Difco), 2% Bacto agar, 50 µM ammonium sulfate, and 2% dextrose.

**Disruption of the C. neoformans GPA1 gene**

The GPA1 gene was amplified from total genomic DNA of strain H99 using primers based on the published sequence of the gene (Tolkaicheva et al. 1994): 5′-ATGGCCGGCTGTATGCTC-TAC-3′ (Primer 1) and 5′-TAAGATACCAAGAGTCGCTA-3′ (Primer 2). The resulting 1.6-kb fragment was cloned into the pCR2.1 TA cloning vector (Invitrogen). The pCnad2Δap plasmid containing the GPA2 gene of C. neoformans (Tofaletti et al. 1993) was digested with Xhol, and the ADE2-containing fragment was blunt-end ligated into the single Ndel site in the GPA1 gene. The purified plasmid was precipitated onto 0.6 µg gold microcarrier beads (Biorad) and biolistically transformed into the ade2 mutant strain M 049 as described previously (Per-fect et al. 1993). Stable transformants were selected on synthetic medium lacking adenine and containing 1 M sorbitol.

**Confirmation of the gpa1 mutant**

**PCR** Genomic DNA was isolated according to the protocol by Perfect et al. (1993). Genomic DNA (50 ng) from each of 50 gpa1::ADE2 transformants was used as a template for PCR, using 50 pg of the primers described above directed against the extreme 5′ and 3′ ends of the GPA1 gene. The PCR reaction was performed using AmpliTaq DNA Polymerase with GeneAmp buffer and the GeneAmp PCR System 9600 (Roche). The reaction conditions were 35 cycles of denaturation at 94°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 6 min. PCR products were analyzed by agarose gel electrophore-sis.

**Southern hybridization** Genomic DNA (10 µg) from candidate gpa1 mutants was digested with HindIII. Electrophoresis, DNA transfer, prehybridization, hybridization, and autoradiography were performed as described (Sambrook et al. 1989). The probe was labeled using the Random Primed DNA Labeling Kit (Boe-hringer Mannheim) and 32p-dCTP (Amer-sham); the template for the probe was the 1.3-kb PCR fragment amplified from cDNA using the above-described primers corre-sponding to the 5′ and 3′ ends of the GPA1 gene.

**Northern hybridization**

Total RNA was isolated from cell suspensions of the GPA1, gpa1, and gpa1 + GPA1 strains grown in liquid YPD or SLAD (as indicated in the text) using the RNasy Midi Kit (Qiagen). Electrophoresis, RNA transfer, prehybridization, hybridization, and autoradiography were performed as described (Sambrook et al. 1989). The probe was labeled using the Random Primed DNA Labeling Kit (Boehringer Mannheim) and 32p-dCTP (Amer-sham); the template for the probe was the 1.3-kb PCR fragment amplified from cDNA using the above-described primers corre-sponding to the 5′ and 3′ ends of the GPA1 gene.

**Mating assays**

Strains of opposite mating type were cocultured on V8 mating medium and incubated at 25°C for 2 weeks. All cultures to be tested for mating were grown on YPD medium for 48 hr at 30°C immediately before inoculation on mating agar. Mating mixtures were analyzed daily for filamentation using a Zeiss Stemi SV 6 dissecting microscope at 10× magnification. Photomicroscopy was performed on representative sectors of the mating mixtures with a Nikon Axiopt-2 microscope with a 20× objective and a 2.5× trinocular camera adapter.

**Capsule induction**

All strains assayed for capsule production were incubated on YPD medium at 30°C for 48 hr before inoculation into LIM + EDDHA. Packed cell volume measurements were performed on cultures after treatment with 10% formalin and normalization to specific cell counts (see Results) using a micro-hemacytometer (Fisher). The normalized cell suspensions were added to heparinized Microhematocrit Capillary Tubes (Fisher, 02-668-66) and the ends sealed with clay. Capillary tubes were spun for 10 min in a Microhematocrit Centrifuge, Model MB (International Equipment Co.). Packed cell volume was measured, analogously to a hematocrit, as the length of the packed cell phase divided by the length within the capillary tube of the total suspension.

**Melanin production**

All strains assayed for melanin production were initially incubated on YPD medium at 30°C for 48 hr. The cultures were then incubated on Niger seed agar for 7 days at 37°C. Phenoloxidase activity was assayed as described previously with minor modifications (Rhodes 1986). Cells were inoculated from fresh cultures grown on YPD agar into liquid YNB (6.7 grams/liter) with 0.1% dextrose and incubated at 37°C for 16 hr in a shaking incubator. Cells were pelleted, washed once with liquid YNB without dextrose, and resuspended in the same medium. Cells were incubated for an additional 5 hr at 37°C, pelleted, washed once with water, and resuspended in 0.05 M sodium phosphate, pH 7.0 (1 ml of sodium phosphate solution for each 100 mg of cells, wet weight). Toluenethanol (1:4, vol/vol), 100 µl per 1 ml of cell suspension, was added to permeabilize the starved cells, and the mixture was vortexed for 90 sec. The cell suspensions were incubated at 30°C for 2 hr either with or without 1 mM caffeic acid. The suspensions were pelleted in a microcentrifuge at 14,000 rpm for 30 sec and the supernatants analyzed in a Beckman DU 640 spectrophotometer at 480 nm. The A480 of samples to which caffeic acid had been added was compared with a sample in which no caffeic acid had been added as the blank.
Virulence assay
Strains were incubated on YPD medium for 48 hr at 37°C and resuspended in phosphate buffered saline (PBS), pH 7.4 (Sigma) at 3 × 10⁶ cells/ml. Twelve New Zealand white male rabbits, which had been intramuscularly injected with 2.5 mg/kg of cortisone acetate (Merck), were intracerebrally inoculated with 0.3 ml of the cell suspensions (four rabbits per strain). Daily cortisone intramuscular injections were continued throughout the course of the experiment. CSF was obtained sterily by cisternal punctures at days 7, 10, and 14 and immediately plated at various concentrations on YPD medium and incubated at 30°C for 72 hr for quantitative analysis. Rabbits were sedated with 10 mg xylazine and 100 mg ketamine given intramuscularly before all cisternal inoculations or withdrawals (Perfect et al. 1993).

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G-protein regulation of mating and virulence

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