EUKARYOTIC CELL, June 2002, p. 366–377 Vol. 1, No. 3
1535-9778/02/$04.00+0 DOI: 10.1128/EC.1.3.366–377.2002

Pheromones Stimulate Mating and Differentiation via Paracrine and Autocrine Signaling in *Cryptococcus neoformans*

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Received 14 December 2001/Accepted 1 March 2002

Cryptococcus neoformans **is a pathogenic fungus with a defined sexual cycle involving haploid *MAT*α and *MAT*a cells. Interestingly, *MAT*α strains are more common, are more virulent than congenic *MAT*a strains, and undergo haploid fruiting in response to nitrogen limitation or *MAT*a cells. Three genes encoding the MFα pheromone were identified in the *MAT*α mating-type locus and shown to be transcriptionally induced by limiting nutrients and coculture with *MAT*a cells. The *MFα1*, *MFα2*, and *MFα3* genes were mutated, individually and in combination. *MAT*α strains lacking MFα pheromone failed to induce morphological changes in *MAT*a cells. Pheromoneless *MAT*α mutants were fusion and mating impaired but not sterile and mated at ~1% the wild-type level. The pheromoneless *MAT*α mutants were also partially defective in haploid fruiting, and overexpression of MFα pheromone enhanced haploid fruiting. Overexpression of MFa pheromone also enhanced haploid fruiting of *MAT*α cells and stimulated conjugation tube formation in *MAT*a cells. A conserved G-protein activated mitogen-activated protein kinase signaling pathway was found to be required for both induction and response to mating pheromones. The MFα pheromone was not essential for virulence of *C. neoformans* but does contribute to the overall virulence composite. These studies define paracrine and autocrine pheromone response pathways that signal mating and differentiation of this pathogenic fungus.**

Cryptococcus neoformans is an opportunistic human pathogenic fungus first identified more than 100 years ago (10, 55). The incidence of disease caused by *C. neoformans* has increased dramatically in the past two decades due to an increasing population of immunocompromised individuals. *C. neoformans* infection results from inhalation of spores or dessiccated yeast cells, which then spread via the bloodstream to infect the central nervous system and cause life-threatening meningoencephalitis. Studies of the pathogenesis of *C. neoformans* have defined two important virulence factors: a large polysaccharide capsule that impairs phagocytosis and promotes intracellular survival, and melanin, a pigmented polymer that ensheaths the fungal cells and serves as an antioxidant to protect from oxidative and nitrosative challenge by macrophages (12, 22, 40, 41, 54).

C. neoformans is a heterothallic basidiomycete with a defined sexual cycle involving haploid cells of two mating types: *MAT*α and *MAT***a** (2, 37). Mating occurs when two strains of opposite mating type are cocultured under certain nutritional conditions, such as nitrogen limitation, and is thought to be regulated by pheromones (18, 45). After cell fusion, a heterokaryotic filamentous mycelium develops. The tips of the filaments differentiate to form a rounded structure, the basidium, where nuclear fusion, meiosis, and sporulation occur. Interestingly, strains of the *MAT*α mating type differentiate by a process termed haploid or monokaryotic fruiting, which involves filamentation, basidia formation, and sporulation as haploids (64). This process occurs to a limited extent in response to nitrogen limitation alone and is dramatically stimulated when *MAT***a** cells are in close proximity (62).

The *MAT*α mating-type locus is also linked to prevalence and virulence of *C. neoformans*. *MAT*α strains are more common than *MAT***a** strains in the environment (38), virtually all clinical isolates are of the *MAT*α mating type, and *MAT*α strains are more virulent than congenic *MAT***a** strains in a murine tail vein injection model (39).

An ~40-kb region of the genome containing part of the *MAT*α locus was previously identified by a difference cloning method (45). One gene encoding a presumptive mating pheromone, *MFα1*, was identified and found to stimulate conjugation tube formation when transformed into *MAT***a** cells. The *C. neoformans* MFα1 pheromone is similar to *Saccharomyces cerevisiae* a-factor and basidiomycetous fungal pheromones, which all have a characteristic CAAX prenylation motif at the carboxy terminus (7, 8, 14, 24, 44, 46, 48). Recent studies have revealed that farnesylation and carboxymethylation directed by the CAAX motif are necessary for the activity of expressed or synthetic MFα1 pheromone (18).

Mating pheromones are known to be essential for the initial recognition and cell-cell fusion steps during mating in both budding and fission yeasts (17, 57, 65). In contrast, in the homobasidiomycetes *Coprinus cinereus* and *Schizophyllum commune*, cell fusion occurs promiscuously and is not regulated by pheromone. In these organisms, mating pheromones control nuclear migration and fusion of the hook or clamp cells that link the filament cells and function to ensure proper nuclear migration (11, 33, 34, 36, 59). In the hemibasidiomycetous plant fungal pathogen *Ustilago maydis*, mating pheromones play roles in both early and late stages of mating, controlling

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TABLE 1. *C. neoformans* strains

Strain	Genotype[a]	Source (reference)
JEC20	*MAT***a**	Kwon-Chung et al. (39)
JEC21	*MAT*α	Kwon-Chung et al. (39)
JEC30	*MAT***a** *lys1*	J. Edman
JEC34	*MAT***a** *ura5*	Moore and Edman (45)
JEC43	*MAT*α *ura5*	Moore and Edman (45)
JEC50	*MAT*α *ade2*	Moore and Edman (45)
JEC53	*MAT***a** *ura5 lys1*	J. Edman
JEC155	*MAT*α *ura5 ade2*	J. Edman
JEC169	*MAT***a** *ura5 ade2 lys1*	J. Edman
JEC171	*MAT***a** *ade2 lys2*	J. Edman
WSC1	*MAT*α *mfα1*::*ADE2 ura5 ade2*	This study
WSC13	*MAT*α *mfα2,3*::*URA5 ura5*	This study
WSC18	*MAT*α *mfα1*::*ADE2 mfα2,3*::*URA5 ade2 ura5*	This study
WSC50	*MAT*α *mfα1*::*ADE2 mfα2,3*::*ura5 ade2 ura5* (FOA^r)	This study
WSC51	*MAT*α *mfα1*::*ADE2 mfα2,3*::*ura5 ade2 ura5* (FOA^r)	This study
WSC56	*MAT*α *mfα1*::*ADE2 mfα2,3*::*ura5 ade2 ura5* (FOA^r) *MFα1-URA5*	This study
WSC129	*MAT*α *gpb1*::*URA5 ura5*	This study
WSC131	*MAT*α *gpb1*::*ura5 ura5* (FOA^r)	This study
RDC21-14	*MAT*α *ste7*::*ADE2 ade2 ura5*	R. C. Davidson et al., unpublished data
RDC46-3	*MAT*α *mfα1*::*ADE2 mfα2,3*::*URA5 ade2 ura5*	This study
RDC46-4	*MAT*α *mfα1*::*ADE2 mfα2,3*::*URA5 ade2 ura5*	This study

[a] FOA^r, resistant to 5-FOA.

conjugation tube formation and cell fusion and also the stability of the heterokaryotic filaments (5, 35, 56).

Here we took a molecular genetics approach to analyze roles of the pheromones in mating, differentiation, and virulence of *C. neoformans*. Our studies reveal that the mating pheromones play a paracrine signaling role, activating morphological changes in mating partner cells that promote cell fusion and conjugation, similar to the role of pheromones in *U. maydis*. Our studies also reveal that the MFα mating pheromone plays an autocrine signaling role, stimulating filamentation and sporulation of haploid *MAT*α cells in response to nutrient limitation. Studies of cell growth and mating in the protozoan ciliate *Euplotes raikovi* have revealed an analogous role for secreted pheromones in regulating mitogenesis via autocrine signaling and mating via paracrine signaling between cells of opposite mating type (6, 49, 61). Taken together, these findings suggest that an ancient autocrine role for pheromones in self-signaling was coopted to play a paracrine signaling role in mating partner recognition in both uni- and multicellular eukaryotes. Alternatively, the unique ability of *MAT*α cells to differentiate via haploid fruiting may have in part evolved via mutations that enable *MAT*α cells to sense and respond to their own mating pheromone.

MATERIALS AND METHODS

Strains. All studies were conducted with congenic serotype D strains derived from JEC20 (*MAT***a**) and JEC21 (*MAT***a**) and their auxotrophic derivatives (30, 39, 45). All strains used are listed in Table 1.

Transformations. Transformation by electroporation was performed by the method described by Edman and Kwon-Chung (20) by using a Bio-Rad gene pulser (480 mV, 25 μF, 800 Ω). This method was used for all pCnTel1-based plasmids, which were cleaved with the rare-cutting meganuclease enzyme I-*Sce*I to reveal the telomeric ends prior to transformation. Biolistic transformations were performed by the method previously described by Toffaletti and Perfect (58) by using a Bio-Rad Model PDS-1000/He Biolistic Particle Delivery System. This method was used for all non-telomere-based plasmids.

Pheromone gene deletions. Deletion alleles were constructed by subcloning flanking DNA as PCR products or genomic subclones and replacing the coding regions with *C. neoformans ADE2* or *URA5* gene cassettes. The *mfα1* pheromone gene deletion construct was made by overlap PCR (31). The *C. neoformans ADE2* gene was cloned in place of the *MFα1* coding region. The deletion fragment was released from the vector and biolistically transformed into the *MAT*α *ade2 ura5* strain JEC155. Transformants were selected on synthetic medium lacking adenine and containing 1 M sorbitol. Ade$^+$ transformants were colony purified, and deletion mutants were identified by PCR analysis. One deletion strain was backcrossed to the *MAT***a** *ade2 lys2* strain JEC171 and a *MAT*α prototrophic *mfα1* deletion strain selected for phenotypic characterization. The *mfα2,3* pheromone gene deletion plasmid was made by inserting the *URA5* gene between a 1.4-kb *Bam*HI-*Xho*I PCR fragment downstream of the *MFα3* gene and a 1-kb *Xho*I-*Kpn*I PCR fragment downstream of the *MFα2* gene to replace the *MFα2,3* genomic region with the *URA5* marker. The *mfα2,3* deletion allele was released and biolistically transformed into the *MAT*α *ura5* strain JEC43 to create the *mfα2,3* deletion strain or into the *mfα1 ura5* deletion strain to create the *mfα1,2,3* deletion strain (WSC18). Transformants were selected on synthetic medium lacking uracil and containing 1 M sorbitol. Transformants were screened by PCR and confirmed by Southern blot. The *MFα1* gene linked to the *URA5* marker was reintroduced into a *ura5* derivative of the *mfα1,2,3* triple mutant strain (WSC50) (generated by selection on 5-fluoroorotic acid [5-FOA] medium) to yield the *mfα1,2,3 MFα1* strain (WSC56).

Northern blot analysis. *C. neoformans* serotype D wild-type *MAT*α and *MAT***a** strains and pheromone deletion strains were grown in liquid yeast extract-peptone-dextrose (YPD) medium overnight at 30°C. The cells were harvested, washed with sterile water, and resuspended in sterile water at 1×10^9 to 5×10^9 cells/ml. Then, 200 μl or 50 μl of *MAT*α wild-type and pheromone deletion strains were spread on V8 or YPD medium. Parallel experiments were performed by spreading the strains mentioned above with the same amount of wild-type *MAT***a** cells as mating mixtures. Plates were incubated at 24°C for 28 h, and the cells were collected and lyophilized. Total RNA was isolated by using the RNeasy Mini Kit (Qiagen) and electrophoresed, transferred, and subjected to Northern hybridization by standard procedures. The *MF*α common open reading frame, the unique *MFα3* 3′-untranslated region, and the *C. neoformans* actin gene were used as probes.

Mating assays. Strains to be subjected to mating assays were first grown on YPD medium for 2 days at 30°C and then cocultured with the wild-type *MAT***a** strain JEC20 on V8 mating medium at 24°C. Matings were scored daily for filamentation by using a Nikon Eclipse E400 microscope. Photomicroscopy was on representative sectors of mating mixes.

Quantative mating assay. 10^7 cells of the *MAT*α prototrophic wild-type, *mfα1,2,3* mutant, and *mfα1,2,3 MFα1* complemented strains were mixed with 2×10^7 cells of the *MAT***a** *lys1 ura5* strain JEC53 in 100 μl of H_2O. Next, 10 μl of these mixtures was spotted in triplicate onto V8 agar medium, followed by incubation for 10 days. The agar plug containing the mating mixture was excised and completely resuspended in 1 ml of sterile water. The suspension was then serially diluted and plated on synthetic medium lacking lysine and containing

5-FOA, which is toxic to *URA5* strains of *C. neoformans*, to select for recombinant basidiospores. The colonies were counted to determine the mating efficiency.

Cell fusion assays. 10^7 cells of the *MAT*α *mfα1,2,3 ura5* auxotrophic mutant strains WSC50 and WSC51 and the wild-type *MAT*α *ura5* auxotrophic strain JEC43 were each incubated separately with the *MAT***a** *ade2 lys2* auxotrophic strain JEC171 or the *MAT***a** *lys1* strain JEC30 on V8 medium at room temperature (~24°C) for 1 to 4 days. An agar plug containing the entire spot of cells was cut out and vortexed vigorously in sterile H_2O. The agar was allowed to settle and dilutions of the liquid cell mixture were plated to yeast nitrogen base (YNB) minimal medium or SD-Ura-Lys medium to select for heterokaryons, diploids, or recombinants that are prototrophic as a result of cell fusion. The number of colonies that grew after 3 days was counted and recorded.

Haploid fruiting assays. Strains were cultured on YPD medium for 48 h, resuspended in sterile water, spotted onto filament agar, and incubated at 24°C for up to 4 weeks in the dark.

Plasmids. The *MFa1* plasmid (pRCD101) was made by PCR amplification from *MAT***a** genomic DNA with the primers JOHE6604 (5′-GAGCTCCGACA AGTCCGG GAA-3′) and JOHE6605 (5′-TTTTATATCTGACCCGGAAGC-3′) based on the published sequences (13). The PCR product was cloned into the pCR2.1 vector by using the Topo TA Cloning Kit (Invitrogen), which contains flanking *Eco*RI restriction sites to create plasmid pRCD99. The insert was subcloned with *Eco*RI into the telomere containing *C. neoformans*/*Escherichia coli* shuttle vector pCnTel1 marked with *URA5* to create plasmid pRCD101. The *MFα1* plasmid (pRCD17) was made by subcloning the 2.1-kb PCR-amplified *MFα1* region from the pCR2.1-based plasmid pRCD1 (18) into the *C. neoformans*/*E. coli URA5* marked shuttle vector pJMM97-3.

β-Galactosidase assays. Samples were prepared by growing the indicated strains on solid medium, and cells were removed from the agar surface by scraping with a sterile glass rod and resuspending them in distilled water. Cells were then permeabilized by vortexing them for 5 min in 4% chloroform. Control experiments showed that the optical density at 600 nm (OD_{600}) measurement of cultures at different densities corresponded to protein determinations in *C. neoformans*. β-Galactosidase assays were then performed by standard *S. cerevisiae* protocols (9). The Miller units for a sample were calculated by the following standard formula: activity = $(1{,}000 \times X)/(\text{assay time} \times \text{volume assayed} \times Y)$ and represent units of activityper minute per milliliter per OD. Value *X* was calculated by performing the standard β-galactosidase assay and measuring the OD_{595} of the resulting samples. *Y* is the OD_{600} of the culture sample. The results shown are representative of several experiments in which similar results were obtained.

***GPB1* gene disruption.** The *gpb1* gene deletion plasmid was made by inserting the *URA5* gene between a 920-bp *Bam*HI-*Eco*RV PCR fragment upstream of the *GPB1* gene open reading frame and a 1,200-bp *Eco*RV-*Pst*I PCR product downstream of the *GPB1* gene to replace the *GPB1* genomic region with the *URA5* marker. This linear *gpb1::URA5* allele was introduced into the *MAT*α *ura5* strain JEC43 by biolistic transformation and then confirmed by PCR and Southern analysis. A *ura5* derivative of the resulting *gpb1* mutant strain was isolated after selection on 5-FOA medium.

Virulence tests. In vivo testing for virulence assays was conducted in 4- to 6-week-old female DBA mice (NCI/Charles River Laboratories) via tail vein injections. Ten mice were infected with 10^7 yeast cells of each strain in a volume of 100 μl via lateral tail vein injection as described previously (15, 25). The mice were fed ad libitum and monitored with twice-daily inspections. Mice that appeared moribund or in pain were sacrificed by using CO_2 inhalation. Survival data from the mouse experiments were analyzed by a Kruskal-Wallis test.

RESULTS

Identification of the *MFα1*, *MFα2*, and *MFα3* pheromone genes. By sequence analysis of the cloned *MAT*α locus, two additional *MF*α pheromone genes were identified in the well-characterized serotype D strain JEC21 (B-4500) and its congenic derivatives. The *MFα2* pheromone gene (GenBank accession number AF305782) is located 18.5 kb away from the *MFα1* gene (GenBank accession number S56460), and the two genes are transcribed in opposite orientations (see also reference 18). A third pheromone gene, *MFα3*, was also identified and is located immediately adjacent to and 613 bp upstream of the *MFα2* gene; the closely linked *MFα2* and *MFα3* genes are divergently transcribed (GenBank accession number AF069982). Similar mapping results have recently been reported by others (32). No other pheromone genes are apparent in the shotgun sequence coverage (12 times the size of the genome) obtained by the *C. neoformans* serotype D genome sequencing project.

Sequence comparison of the coding and flanking genomic regions of the three pheromone genes reveals that the *MFα1* and *MFα2* genes are highly related, whereas the *MFα3* gene is more divergent. The coding regions of the *MFα1* and *MFα2* genes are identical. The *MFα3* gene has three nucleotide changes in the coding region compared to the *MFα1* and *MFα2* genes; one alteration results in the replacement of threonine 9 of the pheromone precursor with alanine. The promoter regions (526 bp) of the *MFα1* and *MFα2* genes share 94.9% identity, compared with only 83.2 and 83.4% identity between the *MFα3* gene and the *MFα1* and *MFα2* genes, respectively. The *MFα1* and *MFα2* genes differ by only one nucleotide in the 200-bp 3′-untranslated region, whereas this region of the *MFα3* gene is completely divergent.

Mutation of the *MFα1*, *MFα2*, and *MFα3* pheromone genes. To determine the roles of the three *MF*α pheromone genes in *C. neoformans* var. *neoformans*, the genes were mutated individually and in combination in congenic serotype D strains. The *MFα1* gene was replaced with the *ADE2* gene, and the linked *MFα2* and *MFα3* genes were replaced with the *URA5* gene (see Materials and Methods). The resulting *mfα1Δ::ADE2* and *mfα2,3Δ::URA5* deletion alleles were introduced by biolistic transformation. Ade^+, Ura^+, and Ade^+ Ura^+ isolates were screened by PCR, and deletion strains were verified by Southern analysis (Fig. 1A). In the *mfα1*, *mfα2,3*, and *mfα1,2,3* deletion strains, the corresponding pheromone genes were deleted (Fig. 1A and Table 1). No additional pheromone genes that are homologous to *MFα1-3* were detected in the *mfα1,2,3* triple mutant strain by Southern blot analysis of total genomic DNA. Genetic crosses and meiotic segregation analysis confirmed that the *mfα1Δ::ADE2* and *mfα2,3Δ::URA5* mutations were linked to each other, to the *MAT*α mating-type locus, and to a defect in mating and that no ectopic integrations of either marker gene had occurred (Table 2).

Induction of *MF*α pheromone gene expression by nutrient limitation and *MAT*a cells. Expression of the pheromone genes was examined by Northern blot to confirm that the pheromone genes had been functionally mutated and to examine conditions regulating pheromone gene expression. Mating of *C. neoformans* is routinely conducted on V8 medium and does not occur on rich medium such as YPD medium. Cells were grown on V8 and YPD medium with or without congenic *MAT***a** mating partner cells. RNA was isolated and analyzed with probes to the open reading frame common to all three *MF*α genes, the unique 3′ region of the *MFα3* gene, or the actin gene as a control (Fig. 1B). The pheromone genes were expressed at a low level when the wild-type MATα strain was grown on V8 medium alone (Fig. 1B). Pheromone gene expression was dramatically induced by the presence of *MAT***a** mating partner cells on V8 medium (Fig. 1B, compare lanes 1 and 5). The *MFα1* pheromone gene was induced in response to mating partner in the *mfα2,3* mutant strain (Fig. 1B, compare lanes 3 and 7), whereas pheromone gene expression was completely abolished in the *mfα1,2,3* triple mutant strain (Fig. 1B). Importantly, the fact that the *MFα3* gene has a unique 3′ sequence allowed us to show with an *MFα3*-specific probe that

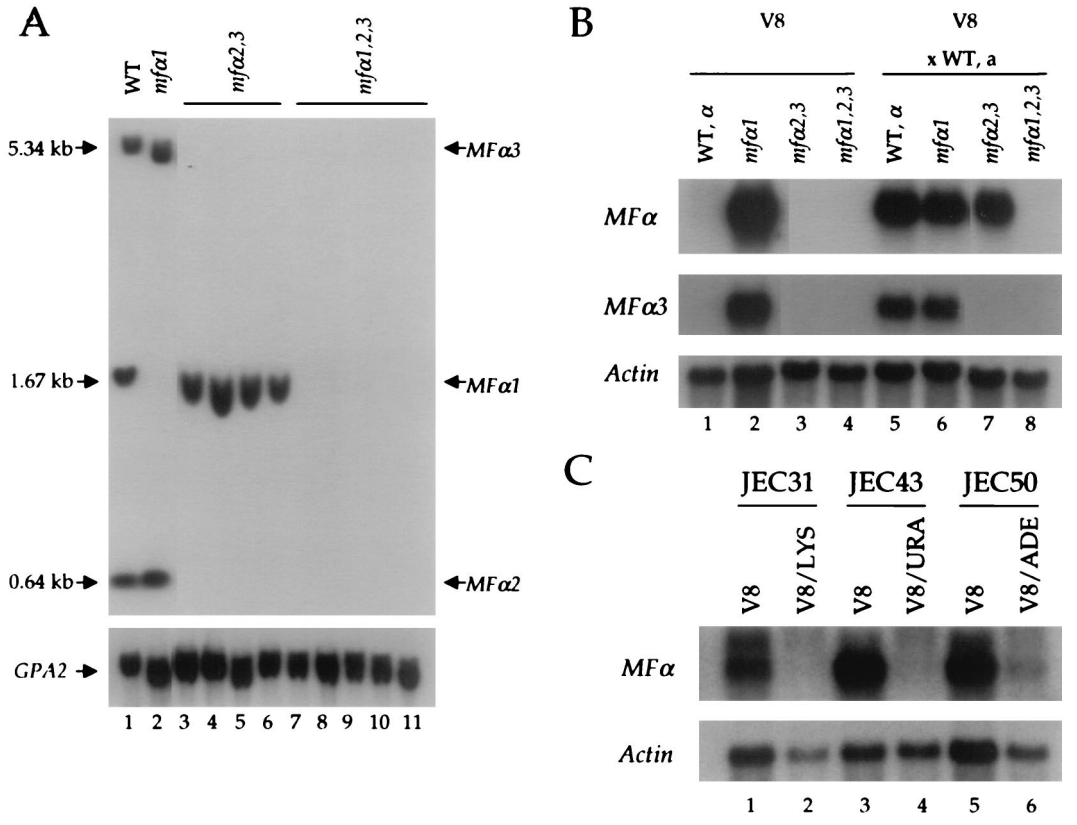

FIG. 1. Disruption and characterization of the *MF*α pheromone genes. (A) The disruption alleles of the *MF*α*1* and *MF*α*2,3* genes were constructed as described in Materials and Methods. Genomic DNA was isolated from the *MF*α*1,2,3* wild-type strain, one *mf*α*1*Δ*::ADE2* mutant strain, four *mf*α*2,3*Δ*::URA5* mutant strains, and five *mf*α*1*Δ*::ADE2 mf*α*2,3*Δ*::URA5* mutant strains; digested with *Xho*I and *Hin*dIII; electrophoresed; and analyzed by Southern hybridization with the common open reading frame of the *MF*α genes as a probe. The *GPA2* gene encoding a *C. neoformans* heterotrimeric G-protein α subunit was used as a control. (B) Total RNA was isolated from the *MAT*α wild-type, *mf*α*1*, *mf*α*2,3*, and *mf*α*1,2,3* strains grown on V8 agar medium in the presence or absence of the wild-type *MAT***a** strain JEC20. Then, 10 μg of total RNA was analyzed with the *MF*α common, *MF*α*3* specific, and actin gene probes. (C) Total RNA was isolated from *MAT*α *lys1* (JEC31), *MAT*α *ura5* (JEC43), and *MAT*α *ade2* (JEC50) auxotrophic strains grown on V8 agar medium or V8 supplemented with 30 μg of lysine, 20 μg of uracil, or 20 μg of adenine/ml, respectively. Next, 10 μg of total RNA was analyzed with the *MF*α common and *actin* gene probes.

this pheromone gene is also induced by a mating partner (Fig. 1B, compare lanes 1 and 5). Furthermore, *MF*α*3* is not expressed in the *mf*α*2,3* and *mf*α*1,2,3* mutant strains (Fig. 1B). These findings reveal that at least the *MF*α*1* and *MF*α*3* genes are induced during coculture with mating partner cells, that the *MF*α*1-3* genes have been functionally deleted in the mutant strains, and that no other homologous pheromone genes are functionally expressed.

Pheromone gene expression was also found to be induced by nutritional limitation. First, a modest level of pheromone expression could be detected when *MAT*α wild-type cells were cultured with *MAT***a** cells in YPD medium, but the level of expression was significantly lower than that observed on V8 medium (data not shown).

Second, surprisingly, expression of the *MF*α*2* and *MF*α*3* genes was readily detected when the *mf*α*1* mutant cells were cultured on V8 medium in the absence or presence of *MAT***a** mating partner cells. In contrast to the wild-type and *mf*α*2,3* and *mf*α*1,2,3* mutant strains, the *mf*α*1* mutant strain is a *ura5* auxotroph, and V8 medium is limiting for uracil and additional nutrients. Similar findings were obtained with *MAT*α *lys1*, *MAT*α *ura5*, and *MAT*α *ade2* mutant strains: *MF*α expression was dramatically induced during culture on V8 medium alone,

TABLE 2. Segregation analysis of an *mf*α*1,2,3* triple mutant strain[a]

Segregant	Marker[b]			*MAT*	Mating efficiency[c]
	Ade	Ura	Lys		
1	−	−	−	**a**	Fertile
2	−	−	+	**a**	Fertile
3	−	−	+	**a**	Fertile
4	−	−	+	**a**	Fertile
5	+	+	−	α	Sterile
6	+	+	−	α	Sterile
7	−	−	+	**a**	Fertile
8	+	+	+	α	Sterile
9	+	+	+	α	Sterile
10	+	+	+	α	Sterile
11	+	+	−	α	Sterile
12	+	+	−	α	Sterile
13	+	+	−	α	Sterile
14	+	+	+	α	Sterile
WSC18	+	+	+	α	Sterile
JEC169	−	−	−	**a**	Fertile

[a] Parents: *MAT*α *mf*α*1*::*ADE2 mf*α*2,3*::*URA5 ade2 ura5* (WSC18) × *MAT***a** *ade2 lys1 ura5* (JEC169).

[b] Growth (+) or no growth (−) on culture medium lacking adenine (Ade), uracil (Ura), or lysine (Lys).

[c] Fertile, wild-type mating; sterile, 1% mating equivalent to the *mf*α*1,2,3* mutant.

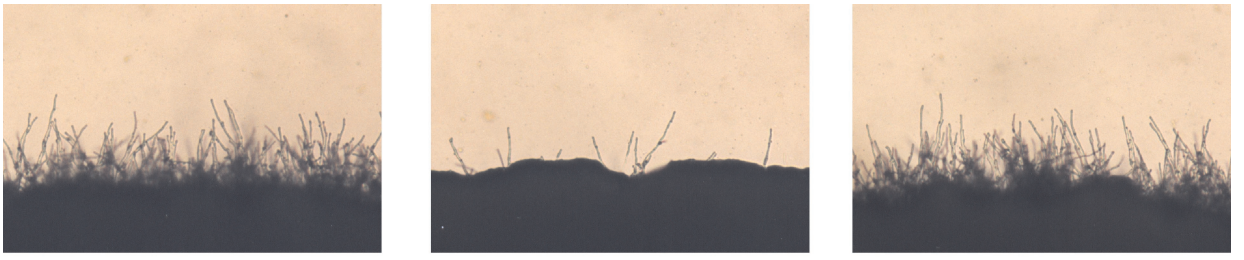

FIG. 2. MFα pheromone promotes but is not essential for mating. The isogenic wild-type (JEC21), *mfα1,2,3* (WSC18), and *mfα1,2,3* plus *MFα1* (WSC56) strains were coincubated with a *MAT***a** wild-type strain (JEC20) on V8 agar mating medium in room light for 2 days at 24°C. The edges of the mating mixtures were photographed at ×100 magnification.

whereas supplementation with lysine, uracil, or adenine repressed *MF*α expression (Fig. 1C). These findings may explain the finding that auxotrophic *C. neoformans* strains mate with enhanced proficiency compared to prototrophic strains. In summary, *MF*α pheromone gene expression is induced by both nutrient limiting conditions and factors secreted by mating partner cells, such as MFa pheromone. These findings are in accord with our recent studies on *MFα1* gene expression by using a reporter gene approach (18).

MF*α pheromone promotes cell fusion and is important but not essential for mating.** The pheromone triple gene deletion mutants exhibited defects in mating when crossed with congenic wild-type serotype D *MATa** cells on V8 medium and the production of mating filaments was monitored by microscopy. In this assay, the *mfα1* and *mfα2,3* mutant strains mated with an efficiency similar to the wild-type *MAT*α strain (data not shown). By comparison, mating of the *mfα1,2,3* triple mutant strain was significantly reduced compared to the wild type, particularly at early time points (Fig. 2). Importantly, the pheromone triple mutant strain was not completely sterile, and some mating filaments, basidia, and basidiospores were still produced. When these filaments were stained with calcofluor white and ethidium bromide, both fused clamp connections and dikaryotic hyphal cells characteristic of mating were observed (data not shown). Because the microscopy-based assay used to monitor mating efficiency is qualitative, we employed a quantitative mating assay that monitors the production of *ura5* LYS^+ recombinant basidiospores (see Materials and Methods). In this assay, the efficiency of mating of the *mfα1,2,3* pheromone mutant strain WSC18 with the *MAT***a** *lys1 ura5* strain JEC53 was reduced ca. 100-fold compared to the congenic wild-type strain JEC21 (Table 3). Mating was restored to the wild-type level in both the filamentation and quantitative mating assays when the wild-type *MFα1* gene was reintroduced into the *mfα1,2,3* triple mutant by transformation (Table 3 and Fig. 2). Recombinant basidiospores that exhibited meiotic recombination for parental markers were also recovered from similar crosses (Table 2).

The role of the *MF*α pheromone in mating was analyzed in further detail by cell fusion and confrontation assays. The *mfα1,2,3 ura5* mutant strains WSC50 and WSC51 exhibited a defect in cell fusion, and the production of prototrophic heterokaryons was reduced 50- to 100-fold compared to the wild-type *MAT*α *ura5* strain JEC43 coincubated with the *MAT***a** *lys1* strain JEC30 or the *MAT***a** *lys2 ade2* strain JEC171 after incubation on V8 medium for 1 to 4 days (see Materials and Methods and data not shown). Thus, the *MF*α mating pheromone plays a role in the initial cell fusion event during mating. The role of pheromone was also examined in confrontation assays. When wild-type *MAT*α and *MAT***a** cells are grown as lines of confronting cells on filamentation agar, *MAT*α cells produce conjugation tubes and haploid fruit (Fig. 3). In contrast, *MAT***a** cells produce fewer conjugation tubes and instead a large number of enlarged, round, refractile cells are observed (2, 18, 62). The *mfα1,2,3* pheromone triple mutant strain failed to induce confronting *MAT***a** cells to produce either conjugation tubes or enlarged round cells (Fig. 3). Thus, the MFα pheromone controls morphological responses of *MAT***a** cells during mating. In addition, the *mfα1,2,3* mutant cells responded more poorly to *MAT***a** cells and formed fewer conjugation tubes and haploid fruited to a more limited extent than did wild-type *MAT*α cells (Fig. 3). These findings suggest that MFα pheromone is required to induce production of the MFa pheromone that then acts on *MAT*α cells. Alternatively, as discussed further below, additional evidence indicates that the MFα pheromone also acts on *MAT*α cells by autocrine signaling.

MFα pheromone promotes haploid fruiting of *MAT*α cells. Surprisingly, the *mfα1,2,3* triple mutant strain was found to have a significant defect in haploid fruiting in response to nitrogen limitation. Wild-type *MAT*α and the isogenic *mfα1,2,3* mutant strain were cultured on filamentation agar for 10 days in the absence of light to enhance haploid fruiting. As shown in Fig. 4A, the wild-type *MAT*α strain haploid fruited under these conditions and produced filaments, basidia, and basidiospores. In comparison, the pheromone triple mutant strain largely failed to differentiate. Some haploid fruiting filaments were observed with the pheromoneless *MAT*α mutant strain upon prolonged incubation but were decreased compared to the wild-type strain. After genetic crosses and isolation of recombinant basidiospores by micromanipulation, the haploid fruiting defect was found to cosegregate with the *mfα1,2,3* mutations (data not shown). Finally, reintroduction of the wild-type

TABLE 3. Quantitative mating analysis

Strain	Total no. of recombinants (dilution)	Calculated avg	% Wild-type mating
Wild type (JEC21)	38 (1:36 dilution) 56 (1:36 dilution) 80 (1:36 dilution)	2,088	100
mfα1,2,3 (WSC18)	16 (undiluted) 19 (undiluted) 21 (undiluted)	19	0.9
mfα1,2,3 MFα1 (WSC56)	40 (1:36 dilution) 72 (1:36 dilution) 39 (1:36 dilution)	1,812	86.7

MFα1 gene into the *mfα1,2,3* mutant restored haploid fruiting (Fig. 4A).

Haploid fruiting was significantly enhanced in the *mfα1,2,3* plus *MFα1* reconstituted strain compared to the congenic wild-type strain (Fig. 4A). We considered two possible explainations. First, overexpression of the MFα1 pheromone itself might enhance haploid fruiting of *MAT*α cells. Alternatively, regulatory sequence elements in the promoter of the *MFα1* gene could titrate an inhibitory factor and enhance expression of other genes that control differentiation. To distinguish between these models, the *MFα1* gene promoter was replaced with the constitutive promoter from the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene (*GPD1*). When the resulting P_{GPD1}-*MFα1* gene fusion was introduced into the

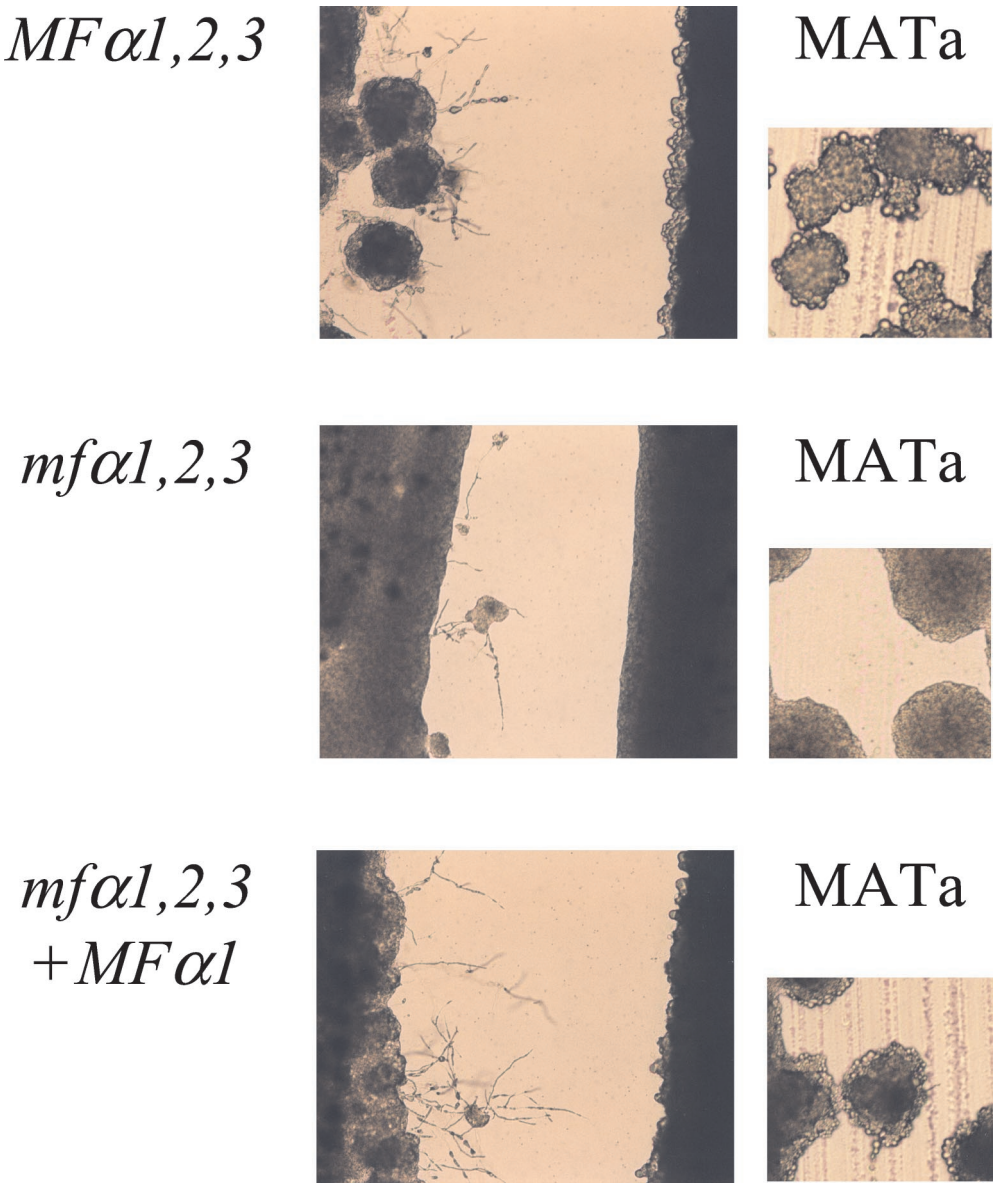

FIG. 3. MFα pheromone regulates morphogenesis of confronting *MAT***a** cells. The *MAT*α wild-type (JEC21), *mfα1,2,3* (WSC18), and *MFα1,2,3 MFα1* (WSC56) strains were incubated in close proximity in confrontation assays with the *MAT***a** strain JEC20 on filament agar and photographed at ×100 magnification (left panels) or at ×200 magnification after 120 h incubation at 24°C (right panels).

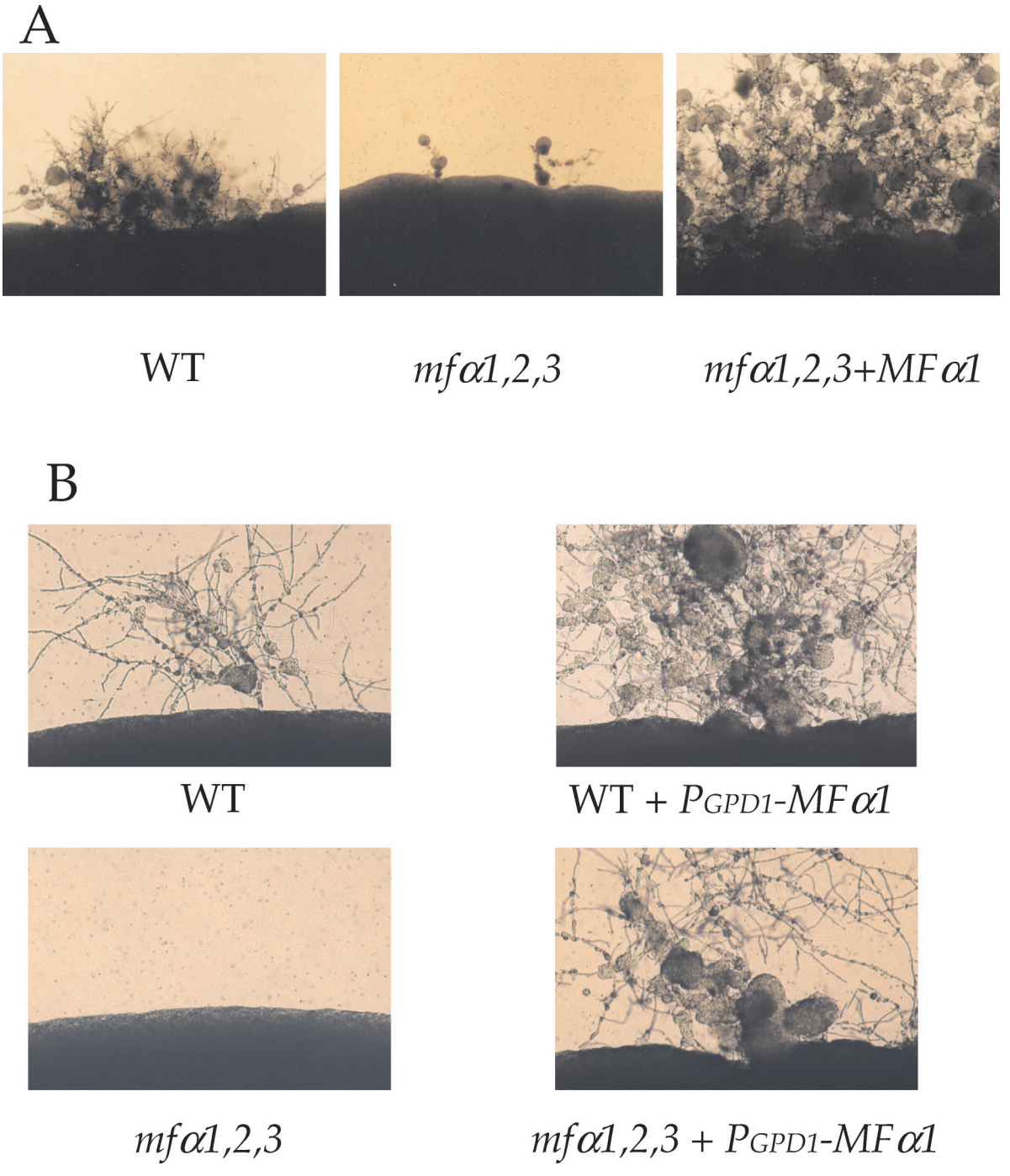

FIG. 4. MFα pheromone stimulates haploid fruiting of *C. neoformans*. (A) The isogenic wild-type (JEC21), *mfα1,2,3* (WSC18), and *mfα1,2,3* plus *MFα 1* (WSC56) strains were incubated on filament agar for 10 days at 24°C in the dark. (B) The isogenic wild type (JEC21), the *mfα1,2,3* mutant strain (WSC17), the *MATα ura5* wild-type strain JEC43 transformed with the P_{GPD1}-*MFα1* gene fusion, and the *mfα1,2,3 ura5* mutant strain (WSC50) transformed with the P_{GPD1}-*MFα1* gene fusion (strain WSC57) were incubated on filament agar for 10 days at 24°C in the dark. The edges of the colony were photographed at ×100 magnification.

wild-type and *mfα1,2,3* mutant strains, haploid fruiting was restored in the *mfα1,2,3* triple mutant and enhanced in wild-type cells by expression of the MFα1 pheromone from a heterologous promoter (Fig. 4B). These observations support the conclusion that the MFα mating pheromone itself signals differentiation of *MATα* cells in response to nitrogen-limiting conditions, possibly via an autocrine signaling pathway.

MFα pheromone directs mating and fruiting via a G-protein–mitogen-activated protein (MAP) kinase cascade. We tested whether overexpression of the MFα1 pheromone en-

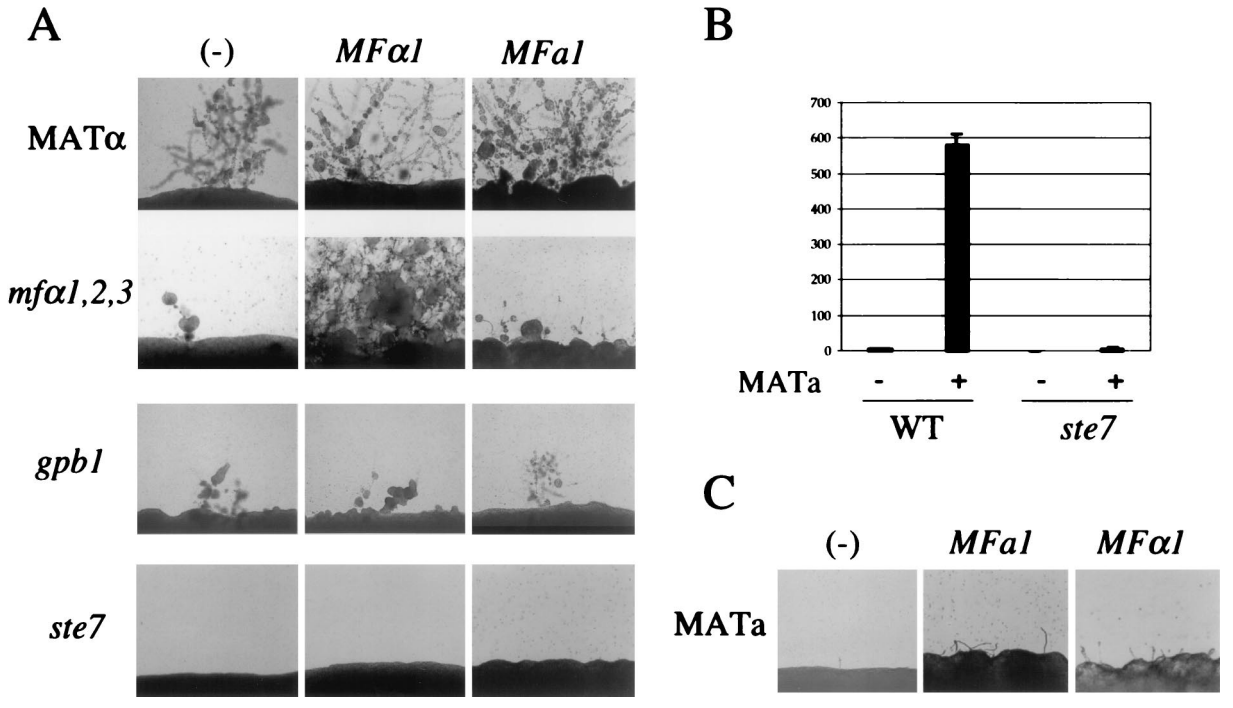

FIG. 5. Pheromones signal haploid fruiting and mating associated gene activation via a conserved G-protein-activated MAP kinase pathway. (A) MFα and MFa pheromones enhance haploid fruiting of wild-type cells but not *gpb1* or *ste7* mutant cells. The isogenic *MAT*α *ura5* wild-type strain (JEC43) and the *mfα1,2,3* (WSC50), *gpb1* (WSC131), and *ste7* (RDC21) mutant strains were biolistically transformed with the MFα1 (pRCD17) or MFa1 (pRCD101) expression plasmids as described in Materials and Methods. Transformants were incubated on filament agar for 5 days at room temperature and photographed at ×100 magnification. (B) The Ste7 MAP kinase is required for activation of MFα pheromone gene expression by *MAT***a** cells. The congenic *MAT*α wild-type strain JEC43 and the *ste7* mutant strain RDC21 were transformed with the *MFα1-lacZ* reporter plasmid pRCD41 (18). Transformants were grown on V8 mating medium for 24 h, and the β-galactosidase activity was measured as described in Materials and Methods and plotted here in Miller units. (C) Both MFa1 and MFα1 pheromones stimulate conjugation tube formation by *MAT***a** cells. The *MAT***a** *ura5* strain JEC34 was transformed with the MFa1 (pRCD101) or the MFα1 (pRCD17) pheromone expression plasmid by biolistics as described in Materials and Methods, and Ura^+ transformants were incubated on filament agar for 2 days at room temperature and photographed at ×100 magnification.

hances haploid fruiting of *MAT*α cells via known components of the pheromone response pathway. To this end, the G-protein β-subunit Gpb1 implicated in regulating mating by pheromone (62) was identified from the congenic serotype D strains JEC21 and JEC20 and replaced with a *gpb1::URA5* mutant allele by biolistic transformation and homologous recombination in the *MAT*α *ura5* strain JEC43. Five transformants identified by PCR and confirmed by Southern blot contained the *gpb1* mutant allele and lacked the wild-type locus (not shown). The *MAT*α *gpb1* mutant strains were sterile when coincubated with the *MAT***a** wild-type strain JEC20 on V8 mating medium (not shown). Haploid fruiting was reduced, but not completely abolished, in the *MAT*α *gpb1* mutant strains, similar to the *mfα1,2,3* pheromoneless mutant (Fig. 5A). When the *MFα1* pheromone was overexpressed from either the *MFα1* or the *GPD1* gene promoter in *ura5* derivatives of the *gpb1* mutant strains, the partial defect in haploid fruiting conferred by the *gpb1* mutation was not suppressed (Fig. 5A and data not shown).

Because haploid fruiting induced by overexpression of the MFα pheromone required the G protein β subunit Gpb1, we next tested whether signaling occurs via a MAP kinase pathway. Members of a putative pathway regulating mating and filamentation have been identified in *C. neoformans* (R. C. Davidson et al., unpublished data), including a homolog of the gene enconding the MAP kinase kinase Ste7 that regulates mating and filamentation in *S. cerevisiae. C. neoformans* serotype D *ste7* mutant strains exhibit a severe defect in mating and haploid fruiting (Davidson et al., unpublished data). When the *MFα1* pheromone was overexpressed from either the *MFα1* or the heterologous *GPD1* gene promoter in *ste7* mutant strains, the defect in haploid fruiting was not suppressed (Fig. 5A and data not shown).

Induced expression of the *MFα1* pheromone gene in response to MFa pheromone or other factors produced by *MAT***a** cells was found to require the Ste7 kinase. Expression was monitored with an *MFα1-lacZ* reporter gene introduced into *MAT*α *STE7* wild-type and *ste7* mutant cells that were cocultured with *MAT***a** cells. *MFα1* gene expression was induced in wild-type *MAT*α cells by *MAT***a** cells, but not in *MAT*α *ste7* mutant cells (Fig. 5B). We note that the basal level of MFα1 pheromone expression in nutrient-limited cells did not require either Gpb1 or Ste7. Thus, *MAT*α *gpb1* and *MAT*α *ste7* mutant cells induced morphological changes in confronting *MAT***a** cells, albeit not to the full extent of wild-type cells, indicating that a reduced level of MFα pheromone is expressed and secreted and is sufficient to mediate paracrine signaling responses in *MAT***a** cells (data not shown). Taken together, these findings indicate that elements of a conserved MAP kinase pathway are required for both induced expression of mating

pheromone and the autocrine effects of the MFα1 pheromone on *MAT*α cells.

MFa pheromone stimulates fruiting of *MAT*α cells and morphogenesis of *MAT*a cells. The MFa pheromone was also found to function in both paracrine and autocrine signaling fashions to regulate morphogenesis and development of both *MAT*α and *MAT***a** cells. A 2.1-kb region spanning the *MFa1* gene (13) was PCR amplified and cloned in the *E. coli*/*C. neoformans URA5* shuttle plasmid pCnTel1. When the resulting MFa1 expression plasmid was introduced into the *MAT*α *ura5* strain JEC43, haploid fruiting was dramatically enhanced to a level comparable to *MAT*α cells grown in confrontation with *MAT***a** cells (Fig. 5A). These findings provide further evidence that haploid fruiting of *MAT*α cells is enhanced in a paracrine fashion by MFa pheromone secreted by *MAT***a** cells.

Most interestingly, when *MAT***a** cells were transformed with the MFa1 pheromone expression plasmid, the formation of conjugation tubes was stimulated (Fig. 5C). The autocrine response of *MAT***a** cells to MFa pheromone was somewhat less marked than that observed when the *MFα1* pheromone gene was introduced into *MAT***a** cells (Fig. 5C; see also references 18 and 45). In summary, while cells of each mating type respond more dramatically to the pheromone produced by the opposite cell type, overexpression of the self pheromone (i.e., MFα in *MAT*α or MFa in *MAT***a**) stimulates an autocrine pheromone response in *C. neoformans*.

The MFa pheromone may in part regulate haploid fruiting of *MAT*α cells by inducing the *MF*α pheromone genes. When the *MFa1* pheromone gene was overexpressed in the *mfα1,2,3* pheromone triple mutant, haploid fruiting was partially but not completely restored (Fig. 5A). The *mfα1,2,3* strain expressing the *MFa1* plasmid formed abundant short filaments like the wild-type strain after 3 to 4 days (data not shown). However, after 10 days, the wild-type strain expressing the *MFa1* plasmid formed abundant filaments whereas the response of the *mfα1,2,3* strain was more modest (Fig. 5A). Thus, the MFα pheromone appears to play a role in filamentation that is in part distinct from that of the MFa pheromone.

The MFa pheromone may regulate haploid fruiting of *MAT*α cells via elements of the conserved G-protein-activated MAP kinase cascade. When the MFa pheromone overexpression plasmid was introduced into the *MAT*α *gpb1* or *ste7* mutant strains, no restoration of haploid fruiting was observed, providing additional evidence that both Gpb1 and Ste7 participate in sensing either pheromone during mating and haploid fruiting.

MFα pheromone modestly contributes to but is not essential for virulence. In previous studies, the *MAT*α locus has been linked to virulence of *C. neoformans* (39). We therefore tested whether the MFα mating pheromones contribute to *C. neoformans* virulence in a murine tail vein injection model (Fig. 6). A total of 10^7 cells of the wild-type strain, the *mfα1,2,3* mutant strain, the *mfα1,2,3 MFα1* reconstituted strain, and two independent *mfα1,2,3* mutant strains obtained after a genetic cross were injected into groups of 10 mice each. DBA mice lacking the C5 component of complement and which are particularly susceptible to lethal infection by the congenic serotype D laboratory strains were used (15, 25, 50–52).

Virulence of the original *mfα1,2,3* mutant strain was modestly attenuated compared to the wild-type *MAT*α strain JEC21 ($P < 0.001$). Two independent *mfα1,2,3* mutant f1 meiotic segregants were also modestly attentuated for virulence compared to the wild-type strain JEC21 ($P = 0.024$ and $P = 0.006$), although not to the same extent as the original *mfα1,2,3* mutant strain. The two independent *mfα1,2,3* mutant f1 segregants were equally virulent ($P = 0.24$), both were more virulent than the original *mfα1,2,3* deletion strain ($P < 0.001$ and $P = 0.003$), and both were less virulent than the wild type. These observations suggest that the virulence defect of the original *mfα1,2,3* mutant strain is attributable to the *mfα1,2,3* mutations and other events that occurred during the construction of this strain. In accord with this interpretation, virulence was not fully restored in the *mfα1,2,3 MFα1* reconstituted strain (data not shown). In summary, we conclude that the MFα pheromone is not essential for virulence, since three independent triple pheromone gene deletion mutant strains were capable of causing 100% lethal infection. This said, infection by three *mfα1,2,3* mutant strains resulted in slower progression to lethal infection compared to the wild type, providing evidence that the MFα pheromone makes a modest contribution to virulence.

DISCUSSION

C. neoformans has a bipolar mating-type system in which haploid *MAT*α and *MAT***a** cells fuse to produce a filamentous heterokaryon. Like the model yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, the mating-type locus directs cells fusion in *C. neoformans* and cells with opposite mating type readily fuse, whereas cells with the same mating type rarely do so (C. Hull and J. Heitman, unpublished results). Our findings indicate that the MFα pheromone controls cell fusion in a way that is similar to the role of mating pheromones in the model yeasts. Our findings also reveal that *C. neoformans* mutants lacking the MFα pheromone exhibit a mating defect but are not absolutely sterile. This is in marked contrast to budding and fission yeasts, in which mating pheromones are absolutely required for cell fusion and mating. On the other hand, these findings are more similar to complex basidiomycetes with multiple sexes, such as *S. commune* and *C. cineurus*, in which cell fusion is completely promiscuous and the pheromones only function later in regulating nuclear migration and clamp cell fusion. In these organisms, because there are literally thousands of mating types, virtually every cell encounter is of two different mating types, and stringent regulation of fusion to prevent like pairings is unnecessary (11, 34, 59). Our findings in *C. neoformans* are most similar to previous studies in the basidiomycetous maize pathogen *U. maydis* in which pheromones function to promote conjugation tube formation and cell fusion during the earliest steps in mating (4, 56).

In all of these basidiomycetes, pheromones also likely function during later steps in mating to mediate clamp or hook cell fusion and ensure the stability of the dikaryon. We found no dramatic defect in filament morphology in matings involving an *mfα1,2,3* mutant strain and wild-type *MAT***a** cells. One possible explanation is that MFa pheromone produced locally by the *MAT***a** nucleus may suffice to drive clamp cell fusion by signaling to the MFa pheromone receptor expressed by the *mfα1,2,3* mutant.

A more surprising finding was that the MFα pheromone

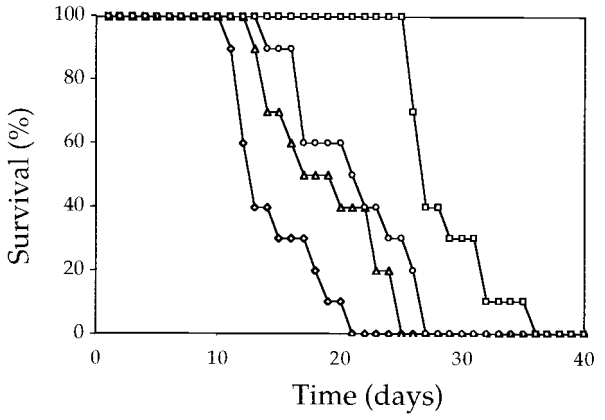

FIG. 6. The MFα pheromone is not essential for virulence. A total of 10^7 cells of the wild-type strain (JEC21), the *mfα1,2,3* mutant strain (WSC18), and two independent *mfα1,2,3* meiotic segregants isolated after a genetic cross (strains RDC46-3 and RDC46-4) were each injected intravenously into groups of 10 C5 complement-deficient DBA mice. Survival was monitored for 40 days and plotted. Symbols: ◇, *MATα*; □, *mfα1,2,3*; △, *mfα1,2,3-1*; ○, *mfα1,2,3-2*.

stimulates *MAT*α cells to differentiate and undergo monokaryotic fruiting, which involves filamentation and sporulation in response to nitrogen limitation. Haploid fruiting of *C. neoformans* was thought to be analogous to pseudohyphal differentiation of *S. cerevisiae* diploid cells in response to nitrogen limitation (26, 64). However, although *S. cerevisiae* pseudohyphal growth is controlled by elements of the MAP kinase cascade that also functions in mating of haploid yeast cells, the pheromones, their receptors, and the coupled G protein are not expressed in diploid cells and do not participate in pseudohyphal growth (42). In contrast, haploid fruiting of *C. neoformans* occurs in haploid *MAT*α cells (64), is activated by the G-protein β-subunit Gpb1 involved in pheromone detection (62), is stimulated by MFα mating pheromone in an autocrine fashion (this study), and can be dramatically enhanced by factors secreted by *MAT***a** cells (62) or expression of the MFa pheromone (this study). These findings suggest that haploid fruiting is normally activated by mating pheromones and plays a role in the early stages of mating. Recent findings reveal filamentous differentiation of haploid *S. cerevisiae* cells is also stimulated by mating pheromones, suggesting pheromone induced filamentation and mating are conserved in divergent ascomycetous and basidiomycetous yeasts (21, 53, 62).

Our findings support a model in which the MFα pheromone enhances differentiation by an autocrine signaling loop that activates the pheromone response pathway. This is surprising because in other fungus mating pheromones act only on cells of the opposite mating type. How might *MAT*α cells respond to their own MFα pheromone? One hypothesis is that the MFα pheromone acts as a partial agonist for the MFa pheromone receptor encoded by the *STE3*α/*CPR1*α gene. The MFα and MFa pheromones are farnesylated carboxymethylated peptides and also share some amino acid identity, particularly at the predicted amino terminus of the mature pheromone (MFα1 [QEAHPGGMTLC*] and MFa1 [EEAYGSGQGPT YSC*]) (13, 18, 45). Thus, the MFα pheromone may bind to the MFa pheromone binding pocket on a common receptor. Similarly, the MFa pheromone acts on both *MAT*α cells by paracrine signaling and on *MAT***a** cells by autocrine signaling. The Ste3a/Cpr1a pheromone receptor expressed by *MAT***a** cells and the Ste3α/Cpr1α receptor expressed by *MAT*α cells share significant amino acid sequence identity (~33%) and may therefore share similar related ligand specificities that contribute to recognition of both self and nonself pheromone ligands. Further studies with synthetic pheromones and heterologous expression of the pheromone receptor genes in *S. cerevisiae*, as has recently been applied to *C. cineurus* and *S. commune* (24, 29, 46), should allow this model to be tested in further detail.

An alternative hypothesis might be that intracellular expression of the mating pheromone precursors alters the processing of other farnesylated proteins involved in signaling. The most likely candidates would be homologs of Ras or the G-protein γ subunit. However, previous studies indicate that these components play or are likely to play positive roles during mating and fruiting (1, 3, 16, 62). Thus, inhibition of their processing should inhibit differentiation in contrast to the stimulation observed upon overexpression of the MFα or MFa pheromone. A second alternative hypothesis is that *C. neoformans* expresses homologs of the mammalian RAMP proteins, which bind to and alter the ligand specificity of G-protein-coupled receptors (43). As yet, no RAMP homolog has been identified in the ongoing genomic sequence project.

Our studies on autocrine pheromone signaling are analogous to previous studies of self-compatible *C. cineurus* mutant strains. In the basidiomycete *C. cineurus*, the unlinked *A* and *B* loci determine mating-type compatibility. The *B* loci encode pheromones and pheromone receptors, and in wild-type strains the pheromones produced by any given *B* locus are not ligands for the receptor encoded by the same locus (28, 29, 48, 60, 63). Olesnicky et al. recently characterized several unusual self-compatible strains with mutations at the *B* locus (47). In one case, a single amino change (R96H) in the second intracellular loop of the Rcb3^6 pheromone receptor enables the receptor to be activated by a normally incompatible pheromone ligand (47). In another case, a single amino acid substitution in the Phb3.2^6 pheromone (F67W) allows the mutant pheromone to activate a normally incompatible receptor (47). Similar studies have been recently reported in the basidiomycete *S. commune*. In this case, mutations in a pheromone ligand caused a change in the cognate receptor, and a two-residue deletion in a receptor prevented recognition of one but not other pheromone ligands (23). Mutations that constitutively activate pheromone receptor signaling, or novel chimeric receptor genes, also confer self-compatibility (27, 46). These studies illustrate how subtle amino acid substitutions in either a pheromone receptor or a pheromone can profoundly alter the ligand-receptor interaction. Our findings in *C. neoformans* suggest the possibility that changes in either the MFa receptor, the MFα pheromone, or both lead to autocrine interactions between the pheromone and receptor that enable *MAT*α cells to sense and differentiate in response to their own mating pheromone.

Our studies on autocrine pheromone signaling are also analogous to previous studies on the regulation of mitogenesis and mating in ciliates. The cosmopolitan soil protozoan ciliate *E. raikovi* exists in several different mating types, and cell-cell interactions during mating are regulated by secreted peptide

mating pheromones (reviewed in reference 6). Importantly, the mating pheromones also regulate mitogenesis in an autocrine signaling fashion in which the secreted pheromone acts on the producing cell (61). Recent studies reveal that the pheromones are expressed in both a membrane-bound and a secreted form, and pheromone recognition involves homotypic interactions between the two forms (49). These findings suggest that the ciliate mating pheromones are similar to mammalian growth factors that drive cell division in an autocrine fashion. Thus, while fungal and ciliate mating pheromones act via distinct signaling mechanisms, there is striking similarity between the autocrine signaling events described in ciliates and our findings in the fungal pathogen *C. neoformans*.

We propose two possible evolutionary scenarios that gave rise to these autocrine signaling mechanisms. In the first model, pheromone signaling originally evolved as an autocrine signaling response that promoted growth, filamentation, and cell fusion of a primordial fungus with a single receptor and a single lipid modified pheromone. During evolution, some fungi evolved cell type signaling specificity in which pheromones came to act on only the receptors expressed by cells of opposite mating type, whereas other organisms retained autocrine signaling. In the second model, pheromones and their receptors evolved early on to act on only opposite mating partners, but in some fungi mutational events occurred to enable autocrine signaling. Mutations of this type have been readily isolated in *C. cinereus* and may explain the autocrine signaling we observe with both MFα and MFa pheromones in *C. neoformans*.

Mating pheromone autocrine signaling may function like the autoinducer factors that control quorum sensing in bacteria. During quorum sensing, bacteria produce and respond to local gradients of small molecules, often homoserine lactone derivatives, in response to population density. The MFα and MFa mating pheromones are hydrophobic farnesylated peptides that likely remain preferentially bound to the membrane of the producing cell. This would provide a mechanism to locally concentrate the pheromone and promote autocrine signaling in response to population density increases in a growing colony. The MFα pheromone genes are induced by nutrient deprivation, which occurs as the colony expands and increasing numbers of cells exhaust the nutrient supply. Autocrine signaling would then stimulate haploid filamentation and sporulation, producing spores that could escape from the colony to forage for nutrients or mating partners.

Our studies also addressed the role of the MFα mating pheromones encoded by the *MAT*α locus in virulence of *C. neoformans*. Several independent mutants lacking the MFα pheromone were capable of causing 100% lethal infections in a murine inhalation model, and thus the MFα pheromone is not strictly essential for virulence. In accord with this finding, the *mfα1,2,3* triple mutant strain had no overt defect in capsule or melanin production and grew normally at 37°C on minimal medium. The virulence potential of the *mfα1,2,3* mutant strains was modestly attenuated compared to the wild type, a finding which is consistent with a minor role for the pheromones in virulence. The *MFα1* gene was previously found to be induced in vivo at 2 to 3 weeks after infection of the central nervous system in a rabbit model of cryptococcal meningitis with a pathogenic serotype A clinical isolate (19). Thus, the *MFα1* gene may respond to nutrient-limiting conditions present in the host, similar to what we observed in our in vitro expression studies. The production of MFα pheromone may subtly alter virulence by acting on the host, on the fungal producing cells by autocrine signaling, or via both mechanisms. We note that, whereas MFα pheromone promotes filamentous differentiation in vitro, during infection *C. neoformans* cells are found virtually exclusively as budding yeast cells (2). Thus, the MFα pheromone might promote other types of autocrine responses in the host that contribute to virulence.

ACKNOWLEDGMENTS

We thank members of the Heitman lab for discussions and John Perfect for his enthusiastic support and encouragement.

This work was supported by NIAID R01 grants AI39115 and AI42159 (to J.H.), and P01 award AI44975 from NIAID to the Duke University Mycology Research Unit. Gary Cox is a Burroughs Wellcome New Investigator in Molecular Pathogenic Mycology. Joseph Heitman is a Burroughs Wellcome Scholar in Molecular Pathogenic Mycology and an associate investigator of the Howard Hughes Medical Institute.

W.-C.S. and R.C.D. contributed equally to this study.

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